PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 :
A61K 39/42, 39/00, 39/21, 39/29, 39/12, 39/13, 31/70

A1

(11) International Publication Number: WO 00/45844

(43) International Publication Date: 10 August 2000 (10.08.00)

(21) International Application Number: PCT/US00/02754

(22) International Filing Date: 2 February 2000 (02.02.00)

(30) Priority Data:

09/243,753	3 February 1999 (03.02.99)	US
09/493,769	28 January 2000 (28.01.00)	US

(71)(72) Applicants and Inventors: LORI, Franco [IT/US]; 4400 East West Highway, #1126, Bethesda, MD 20814 (US). LISZIEWICZ, Julianna [DE/US]; 4400 East West Highway, Bethesda, MD 20814 (US).

(74) Agent: LOOPER, Valerie, E. The Law Offices of Valerie E. Looper; 11726 Lightfall Court, Columbia, MD 21044 (US).

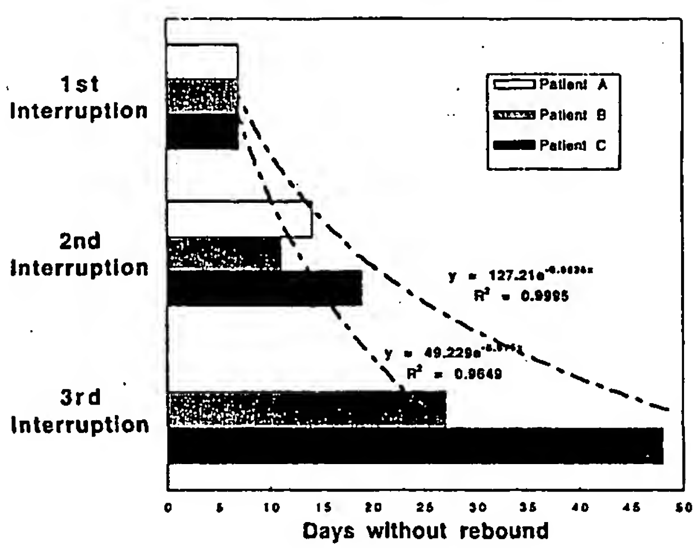
(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: USE OF HYDROXYUREA AND A REVERSE TRANSCRIPTASE INHIBITOR TO INDUCE AUTOVACCINATION BY AUTOLOGOUS HIV

Intermittent therapy delays rebound of HIV



(57) Abstract

A method for inducing autovaccination by autologous human immunodeficiency virus is disclosed comprising the step of administering to said cells a combination of compounds selected from the group consisting of hydroxyurea, a nucleoside analog, and, optionally, a protease inhibitor, and further comprising the steps of stopping treatment and restarting treatment under controlled conditions.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

Use of Hydroxyurea and a Reverse Transcriptase Inhibitor to Induce Autovaccination by Autologous HIV

Field of the Invention

The present invention relates generally to the field of treatment of human beings with Human Immunodeficiency Virus (HIV) infections. The inventors have found that drug combinations of hydroxyurea (HU), one or more reverse transcriptase inhibitors, and optionally, one or more protease inhibitors can be administered intermittently without losing effectiveness as viral population suppressants, even in cases where patients have developed genotypic resistance to the reverse transcriptase inhibitor. The drug combinations can be used in an autologous vaccination technique to enhance a patient's immune system response, thereby allowing long-term, perhaps very long-tem, remission of the disease.

Background of the Invention

Viruses are microorganisms that depend, to some degree, on host cell components for their growth and replication. Viral infection and replication in host cells generally results in disease, whether the host is an animal or plant. Human diseases caused by viral infections include the acquired immunodeficiency syndrome (AIDS) and hepatitis. A general discussion of this field is presented in *Fundamental Virology, Second Edition*, (ed. B. N. Fields, D. M. Knipe, R. M. Chanock, M. S. Hirsh, J. L. Melnick, T. P. Monath, and B. Roizman, Raven Press, Ltd., New York, N.Y. 1991).

Retrovirus Replication

Retroviruses comprise a large family of viruses that primarily infect vertebrates. Many diseases, including the induction of some tumors, are associated with retroviral infection (see *Fundamental Virology*, *supra*, pp. 645-708). All retroviruses, regardless of their clinical manifestations, have related structures and modes of replication.

Retroviruses contain an RNA genome that is replicated through a DNA intermediate. Inside the cell, the viral genome serves as a template

for the synthesis of a double-stranded deoxyribonucleic acid (DNA) molecule that subsequently integrates into the genome of the host cell. This integration occasionally results in the induction of a tumor in the infected host organism. Following integration, a complex sequence of events leads to the production of new viral particles, or progeny virions, which are released from the infected cell.

Early in the retroviral life cycle, the RNA genome is copied into DNA by the virally encoded reverse transcriptase (RT). This enzyme can use both RNA and DNA templates, thereby producing the first strand of DNA (the negative strand) from the infecting RNA genome and a complementary second strand (the positive strand) of DNA using the first DNA strand as a template. To synthesize these DNA strands, the RT utilizes cellular substrates called deoxynucleoside triphosphates (dNTP).

Human retroviruses can be grouped into the leukemia viruses (HTLV type viruses) and the immunodeficiency viruses (HIV type viruses). HTLV infection may lead to one form of leukemia. Acquired immunodeficiency syndrome (AIDS) is caused by a form of HIV, with HIV-1 being more virulent than HIV-2. Both HTLV and HIV infect peripheral blood lymphocytes (PBL).

HIV Infection

HIV-1 was first identified as the causative agent of AIDS in 1983. The AIDS pandemic is now one of the most serious health problems worldwide. Catastrophic medical and social consequences are likely to extend into the next century. The World Health Organization (WHO) has estimated that between eight and ten million people are currently infected with HIV, and that approximately ten times as many individuals will be affected in the next decade. The large pool of HIV carriers makes the development of effective antiviral treatments a medical priority.

The initial HIV-1 infection may occur without accompanying symptoms, but most of the patients experience an acute HIV syndrome within 2 to 6 weeks of exposure to the virus. This syndrome is characterized by fever, headaches, sore throat with pharyngitis, generalized lymphadenopathy and rashes. During this phase the virus replicates abundantly and is detectable in the blood.

The humoral arm of the immune system begins to respond to the infection during this phase. Antibodies to specific proteins associated with

HIV-1 begin to appear in the serum between 2-12 weeks after primary infection. The sequence of appearance of these antibodies can be followed by the Western blot test, which detects the serum antibodies that bind to specific viral proteins. A positive Western blot response to the proteins identified as gp160, gp120, p65, p55, gp41, p32, p24 and p18 demonstrates that antibodies to a signature group of HIV-1 proteins are being produced. The process of change from negative for all the proteins to positive for the entire set is referred to as seroconversion. It has recently been demonstrated that during seroconversion there is a high level of virus present in the blood.

The cellular arm of the immune response also becomes active during seroconversion. That is, various types of immune system cells such as T-cells learn to recognize and destroy infected cells (Borrow et al. Nature Medicine 3:(2) 212-217, 1997; Goulder et al. Nature Medicine 3:(2) 205-211, 1997). However, the number of immune system cells known as CD4+ T-cells falls from a normal amount of $1000/mm^3$ to about $500/mm^3$.

The combination of humoral and cellular immune response together typically causes a decline of viral load in body fluids, or viremia, which ends the acute primary infection phase. In the absence of antiviral therapy, the immune system can partially control viremia, so that the numbers of viral particles in the body drop somewhat, but do not disappear entirely. When the viremia decreases in the blood, the CD4+ T-cell number rises, but absent effective treatment, the T-cell population never fully recovers to the normal level. Over time, the immune system eventually becomes exhausted. When that occurs, viremia can increase. Further, infections from other sources invade the body, and the immune system is less well equipped to handle them. These opportunistic infections are typically the immediate cause of death of the individual.

Viral load, measured as HIV-1 RNA, has been called the best available indicator of disease progression and reduced concentration of HIV-1 in various tissues and fluids in response to antiretroviral therapy is predictive of improved prognosis (Mellors, J.W. et al. Science 272(5265) 1167-1170, 1996).

Antiviral Therapies

There is a critical need to develop effective drug treatments to combat RT-dependent viruses such as HIV. Such efforts were recently

urged in the United Kingdom-Irish-French Concorde Trial conclusions which reported that the nucleoside analog zidovudine (AZT), a mainstay in the treatment of patients infected with HIV-1, failed to improve the survival or disease progression in asymptomatic patients. Other nucleoside analogs, such as 2',3'-dideoxyinosine (ddI) are currently under evaluation. The effects of ddI on disease progression and patient survival endpoints have not been adequately investigated. Non-competitive HIV-1 RT inhibitors and HIV-1 protease inhibitors have also been recently developed. These materials have different antiviral activities and pharmacokinetics properties, but they all directly target HIV-1 proteins. Despite the high efficacy of these compounds, the initial *in vitro/in vivo* testing has been characterized by the rapid onset of variants of HIV-1 resistant to these drugs. These drug-resistant variants, or escape mutants, retain their virulence, and appear to play a major role in the virus' ability to eventually overwhelm the human immune system. A peculiarity of HIV is that it demonstrates an extremely high rate of both reproduction and mutation. As a direct consequence, drugs which demonstrate what would in any other context be regarded as high efficacy (99.9% reduction of viral load in plasma) have not been shown to be able to eliminate the virus from an individual's system. Further, an individual may have undetectable levels of virus as measured by viral load in plasma and biopsy of lymph nodes during treatment, and yet remain infected: once treatment is stopped, the viral rate of replication increases, and the viral load rebounds. In an attempt to obtain greater accuracy, the present inventors have used the most sensitive test methods available. Further, testing of lymph nodes is done by extracting an entire node as opposed to a biopsy sample.

Since escape mutants play such a significant role in the development of the disease, a major focus in current efforts to find a mode of treatment for AIDS is to develop strategies that feature multiple, highly effective, concurrent attacks on HIV in an effort to completely eradicate the virus from an individual's system. The only conclusive proof of effectiveness will be lack of rebound of the viral load in the individual's tissues over time.

At present, there is much interest in trying various combinations of two, three or even four drugs simultaneously. However, it has been admitted that the number of "promising" drugs is "almost astronomical". See *Antiviral Therapy for Human Immunodeficiency Virus Infections*, E. De

Clercq, Clinical Microbiology Reviews, **8**:2, Am. Soc. for Microbiology (Apr. 1995) .

A triple drug combination involving the use of AZT, 3TC and protease inhibitors has been suggested for the treatment of HIV-1 infection and eradication of the virus. The efficacy of this combination is thought to originate from the potency of the protease inhibitors and the mechanism of action of the AZT/3TC combination in inhibiting the rebound of resistant mutants. However, neither the protease inhibitors nor 3TC easily penetrate to certain organs such as lymph nodes and the brain, and the combination of protease inhibitor, AZT and 3TC apparently does not completely eradicate HIV-1 in macrophages or in quiescent cells, which are major reservoirs of HIV-1. Further, patients who have interrupted therapy using AZT, 3TC and protease inhibitors and then rebounded cannot be as effectively treated with the same combination because they develop resistant mutants. Finally, the protease-containing combinations without hydroxyurea have shown at best, response rates of 80-90% and 53% "failure" - a combined figure including people who never responded to therapy, those who could not tolerate side effects, those who responded initially but later saw a return of detectable virus, and those who had difficulty adhering to the strict dosing regimens required by the drugs. See Project Inform Perspective 23:1-3, November 1997.

Hydroxyurea has been widely used over the last three decades for the treatment of leukemia, sickle cell anemia, and has more recently been suggested for use in the treatment of HIV infections, see *Hydroxyurea as an Inhibitor of Human Immunodeficiency Virus-Type 1 Replication*, F. Lori, et al., Science **266**:801-805 (1994); possibly in combination with a nucleoside analog such as AZT, ddI, or ddC, although it has been admitted that clinical trials using hydroxyurea alone or in combination with nucleoside analogs will be essential to assess the actual impact of use of hydroxyurea in HIV-1 impacted patients. *Hydroxyurea and AIDS: An Old Drug Finds a New Application?* F. Lori and R. Gallo, Aids Research and Human Retroviruses Vol. 11, No. 10 Mary Ann Liebert, Inc. (1995). EPO patent publication 94918016.0 filed May 17, 1994 and corresponding to USSN 08/065,814, filed May 21, 1993, which is incorporated herein as if set forth in full, describes the administration of hydroxyurea in combination with ddI, and has reported a therapeutic effect in that CD4+T-cell populations stabilized or increased in human volunteers. This result does not

necessarily demonstrate that any of the individuals were cleared of the virus, because when any patient has stopped any therapy to date, an immediate rebound of viral load has occurred.

Hydroxyurea and nucleoside analogs such as ddl have potent effects on resting cells and macrophages (ref. Lori, PNAS 93 and Science 94 ; Goa-Wy; Agbaria R., Driscoll, J.S.,; Missuya, H.; J. Biol-Chem. 1994 Apr 29; 269(17); 12633-8 ; AU: Gao-W.Y.; Shirasaka, T.; Johns, D.G.; Broder, S.; Mitsuya, H.; J.Clin. Invest. 1993 May: 91(5): 2326-33) which one can speculate represents the route of initial infection during sexual, parenteral and vertical transmission, (1. SO: Science, 1993 Aug 27:261(5125); 1179-81. 2. SO: J. Clin. Invest. 1994 Nov: 94(5): 2060-7 4 . SO: J. Clin. Microbiol. 1995 Feb; 33(2); 292-7 , 5. S: AIDS. 1995 May; 9(5): 427-34 ; 6. SO: J. Exp. Med. 1996 Apr 1; 183(4): 1851-6), and this could represent an advantage of the proposed combination.

Protease inhibitors have received much attention recently in the press as being useful in combination with other drugs such as nucleoside analogs, most especially the combination of AZT and 3TC, to inhibit HIV replication enough to yield improved quality of life for AIDS patients. It has been reported that the viral load in the plasma of such patients is greatly reduced, but not necessarily eliminated, and that whenever treatment has been stopped, the patients have experienced an increase in viral load (rebound) within a matter of 2-3 days. Further, viral rebound has been shown to occur in patients even when treatment is being continued.

The inventors have reported their discovery that a combination of hydroxyurea, a nucleoside analog, and a protease inhibitor can be used to inhibit HIV in human beings, with greatly improved results in that viral rebound may be delayed for at least three to eight weeks or more. These results indicate that the triple combination which includes hydroxyurea may be used for the treatment of HIV infection. Again, this combination takes advantage of the potency of the protease inhibitors, especially Indinavir. The inventors have found that the double combination of hydroxyurea and a reverse transcriptase inhibitor can also be used, without the addition of a protease inhibitor, for long-term treatment of HIV infections, without provoking viral rebound, and also that use of an immune system stimulant such as a vaccination known to activate quiescent cells may be useful. See USSN 09/048,886, "Method of Inhibiting Human Immunodeficiency Viruses using Hydroxyurea and a Reverse Transcriptase. Inhibitor", and

USSN 09/048,576 "Method of Rendering a Human Immunodeficiency Virus Population Replication Incompetent *in vivo* both filed 26 March 1998. The inventors have now confirmed that the present drug combinations can be used in an autologous vaccination technique. (See corresponding USSN 09/243,753 filed 3 February 1999 and Docket No. 7026, filed 28 Jan 2000. All the above patent applications are incorporated by reference herein as if set forth in full.) That is, the virus that has already infected the individual can be manipulated to act as a vaccine which generates a HIV specific immune response. This immune response allows the individual's body to control the rate of HIV replication after drug therapy is withdrawn. In essence, drug treatment can be stopped and restarted under controlled conditions to stimulate a vigorous immune response in the patient to an existing HIV infection.

Summary of the Invention

It is an object of the present invention to provide a method of inhibiting the replication of retroviruses such as HIV-1, HIV-2, HTLV-1 and HTLV-2 and other reverse transcriptase-dependent viruses such as hepatitis B virus in human cells. However, since the invention describes a new autovaccination technique using an autologous virus, it is not limited to reverse-transcriptase dependent viruses. The invention can be used with all pathogens which produce very large numbers of virus particles and exhaust the immune system. A further object of this invention is to provide a treatment for HIV infections that reduces the presence of the virus in both plasma and the lymphoid system, and which inhibits viral rebound after cessation of treatment. It is yet a further object of this invention to provide a method of treating HIV infection which is effective in the very early, as well as later, stages of infection. Yet another object of this invention is to provide a treatment for HIV which relatively less expensive and has relatively low toxicity, therefore increasing its suitability for widespread use in a large population. An even further object of this invention is to provide a method of activating quiescent cells harboring integrated viral DNA under controlled conditions for the purpose of eliminating the integrated viral DNA. Yet another object of this invention is to enhance a patient's immune system response using autologous vaccination techniques.

The present inventors have found that the hydroxyurea (HU) in combination with a reverse transcriptase inhibitor such as 2',3'-

dideoxyinosine (ddI) alone can be used to reduce the level of viral load in the blood to undetectable levels (less than 500 copies per mL), and that such treatment can be sustained over long periods of time. An advantage of the present invention is that it can be used very early after infection, as well as after seroconversion. A further advantage is that it includes combinations that have relatively low toxicity, and may be suitable as a long-term treatment for chronic infection for a wide range of individuals. Yet another advantage is that, in addition to reducing the viral load in plasma and in the lymph nodes to undetectable levels, the present invention has been shown to inhibit viral rebound after treatment is stopped. Further, the present invention includes a drug regimen that can be stopped and then restarted without losing effectiveness against human immunodeficiency virus populations. Yet another advantage is that an alternative mode of treatment may be made available to individuals who are failing protease inhibitor therapy.

These and other objects and advantages of the present invention will become apparent through the text and examples herein.

Brief Description of the Drawings

Figs. 1-3 are the original Western Blot test results corresponding to the data in Tables 1-3, respectively.

Fig. 4 is a timeline showing viral load for one patient.

Figs. 5 and 6 compare the response of patients to ddI alone and ddI plus hydroxyurea. Fig. 5 shows virus levels in the blood over time, and Fig. 6 shows CD4 cell counts over the same time frame.

Fig. 7 describes data derived for thirty-six patients for a 40 week study using HU and ddI therapy.

Fig. 8 describes data derived from a follow-up on 12 patients, for an average of 28 months using HU and ddI therapy.

Figs. 9 and 10 compare the response of patients to combinations of ddI, d4T and a placebo with ddI, d4T, and hydroxyurea. Fig. 9 shows virus levels in the blood over time, and Fig. 10 shows CD4 cell count over time. A change occurs at the 12 week mark where patients "nonresponsive" to the placebo combination replaced the placebo with hydroxyurea.

Fig. 11 is a bar graph showing the average plasma viremia for a group of 12 patients treated with hydroxyurea and ddI over a period of 112 weeks.

Fig. 12 shows the percentage of naive CD8 and CD4 lymphocytes in normal, uninfected people, HIV patients treated with Hydroxyurea and ddI, and untreated HIV patients.

Fig. 13 compares the percentage of committed lymphocytes as CD8+CD28+ lymphocytes and percentage of activated CD8 lymphocytes for the same group as in Fig. 12.

Fig. 14 compares the p24 stimulation index for the same group as in Fig. 12.

Fig. 15 is a time line showing viral load and breaks in treatment for one patient, BM, in a format consistent with Figs. 16-19. This is the same information found in Fig. 4, except that day zero is defined as the date of infection rather than first day of treatment.

Figs. 16-18 time lines showing viral load and breaks in treatment for three monkeys.

Fig. 19 is a consolidated graph of the level of viremia over time for the same three monkeys.

Fig. 20 shows the change in time to viral rebound in three human patients with more than one interruption in drug therapy.

Fig. 21 shows viremia before, during, and after the first structured treatment interruption (STI) of the PANDA cohort treated with hydroxyurea and didanosine. Weeks = weeks after initiation of hydroxyurea and didanosine treatment.

Fig. 22 shows the percentages of STI failure in hydroxyurea-didanosine treated patients (PANDAs) and HAART controls. Failure is defined as viremia rebound > 10,000 copies/ml (in two consecutive tests) or CD4 count decrease below 200 cells/mm^3.

Fig. 23 compares the viremia profile in hydroxyurea-didanosine treated patients (PANDAs) and HAART controls during STI. Average viremia was calculated from all (n=9) PANDAs throughout the 8 weeks of STI. Average viremia was calculated from all (n=8) HAART patients at week 0 and week 2 and from seven HAART patient at week 4 and 6 (since one patient had to restart therapy at week 2). Average viremia of the HAART patients is not shown at week 8, since most of these patients had already restarted therapy at that time.

Figs. 24-26 are the immunological characterization of hydroxyurea-didanosine treated patients (PANDAs) and HAART controls during STI. Fig. 24 shows CD4 count, Fig. 25 shows CD8 count, and Fig. 26 shows CD4/CD8 ratio. Averages were calculated from all (n=9) PANDAs throughout the 8 weeks of STI. Averages were calculated from all (n=8) HAART patients at week 0 and week 2 and from seven HAART patient at week 4 and 6 (since one patient had restarted therapy at week 2). Averages of the HAART patients are not shown at week 8, since most of these patients had already restarted therapy at that time.

Fig. 27 shows the viral load data for 29 monkeys infected with SIV and treated with continuous HAART and STI various drug combinations.

Fig. 28 shows the viral load data for 3 groups of monkeys infected with SIV. The control group was untreated, and the other two groups were treated with either HAART or one of several intermittent therapies based on ddI+PMPA+HU.

Fig. 29 shows the CD4 counts for 29 monkeys infected with SIV at initiation of therapy, during therapy, and 41 days after cessation of therapy.

Detailed Description of the Invention

Hydroxyurea is one of many inhibitors of ribonucleotide reductase, an enzyme known for catalyzing the reduction of ribonucleoside diphosphates to their deoxyribonucleoside counterparts for DNA synthesis. In the present invention, Hydroxyurea inhibits viral replication, and also acts to down-modulate the immune system. Another material that inhibits viral replication and down-modulates the immune system is cyclosporine, a cyclophilin inhibitor. Other ribonucleotide reductase inhibitors include guanazole, 3,4-dihydroxybenzo-hydroxamic acid, N,3,4,5-tetrahydroxybenzimidamide HCl, 3,4-dihydroxybenzamidoxime HCl, 5-hydroxy-2-formylpyridine thiosemicarbazones, and n-(N)-heterocyclic carboxaldehyde thiosemicarbazones, 4-methyl-5-amino-1-formylisoquinoline thiosemicarbazone, N-hydroxy-N'-amino-guanidine (HAG) derivatives, 5-methyl-4-aminoisoquinoline thiosemicarbazone, diaziquone, doxorubicin, 2,3-dihydroxylbenzoyl-dipeptides and 3,4-dihydroxylbenzoyl-dipeptides, iron-complexed 2-acetylpyridine 5-[(2-chloroanilino)-thiocarbonyl]-thiocarbonohydrazone (348U87), iron-

complexed 2-acetylpyridine-5-[(dimethylamino)thiocarbonyl]-thiocarbonohydrazone (A1110U), 2'-deoxy-2'-methylenecytidine 5'-diphosphate (MdCDP) and 2'-deoxy-2', 2'-difluorocytidine 5'-diphospahte (dFdCDP), 2-chloro-9-(2-deoxy-2-fluoro-ß-D-arabinofuranosyl)-adenosine (Cl-F-ara-A), diethyldithiocarbamate (DDC), 2,2'-bipyridyl-6-carbothioamide, phosphonylmethyl ethers of acyclic nucleoside analogs, [eg. diphosphates of N-(S)-(3-hydroxy-2-phosphonylmethoxypropyl and N-2-phosphonylmethoxyethyl) derivatives of purine and pyrimidine bases], nitrosourea compounds, acylclonucleoside hydroxamic acids (e.g., N-hydroxy-n-(2-hydroxyethoxy)-1(2H)-pyrimidineacetamides 1-3, and 2-acetylpyridine 4-(2-morpholinoethyl)thio-semicarbazone (A723U)).

Hydroxyurea has been widely used in cancer therapy as a broad-spectrum antineoplastic drug (R. C. Donehower, *Seminars in Oncology* 19 (Suppl. 9), 11 (1992)). Hydroxyurea is readily absorbed after oral ingestion, rapidly distributed in the body fluids, including the cerebrospinal fluid, and enters cells efficiently by passive diffusion (*Id.*). Its toxic effects are less profound and easier to control than other chemotherapeutic drugs (*Id.*).

In human chemotherapy, hydroxyurea is currently administered using two basic schedules: (a) a continuous daily oral dose of 20-40 mg per kg per day, or (b) an intermittent dose of 80 mg per kg per every third day. Either schedule could be used in the treatment of viral infections. Given the present invention, lower dosages of hydroxyurea may also be effective in treating HIV infections. The presently preferred dosage range for use of hydroxyurea in treating HIV infections is 800-1500 mg per day, which can be divided over a 24 hour period, for example as 300-500 mg three times a day (TID), 500 mg twice a day (BID), or 1,000 mg once a day (QD), assuming an adult weighing about 70 kg. When the patient's weight is over 60 kg, 400 mg TID is preferred, for those under 60 kg, 300 mg TID is preferred. Hydroxyurea is classified as a mildly toxic drug and does not cause immunosuppression. Myelotoxicity is hydroxyurea's dose-dependent toxicity. However, such toxicity can be easily monitored and it is constantly and rapidly reversible after decreasing the dose or suspending the treatment (Donehower, R.C., *Semin. Oncol.* **19**:11 (1992). By monitoring simple parameters such as neutrofils, platelets and red blood cell counts, hydroxyurea can be administered for years,and sometimes for decades.

A second member of the combination of the present invention is a reverse transcriptase inhibitor. Examples include nucleoside analogs,

such as the 2',3'-dideoxyinosine (ddI)(available as Videx® from Bristol Myers-Squibb). Nucleoside analogs are a class of compoounds known to inhibit HIV, and ddI is one of a handful of agents that have received formal approval in the United States for clinical use in the treatment of AIDS. See Clinical Microbiology Reviews, Supra, p. 200. Like zidovudine (3'-azido-2',3' -dideoxythymidine or azidothymidine [AZT] available from Glaxo Wellcome), zalcitabine (2',3' - dideoxycytidine [ddC] available as Hivid® from Hoffman-La Roche), lamivudine 2'-deoxy-3'-thiacytidine [3TC](Epivir® available from Glaxo Wellcome), lodenosine (F-ddA available from US Biosciences and stavudine (2',3' -didehydro-2',3'-dideoxythimidine [D4T] available as Zerit® from Bristol Myers-Squibb), ddI belongs to the class of compounds known as 2',3' - dideoxynucleoside analogs, which, with some exceptions such as 2',3'-dideoxyuridine [DDU], are known to inhibit HIV replication, but have not been reported to clear any individual of the virus. Other nucleoside reverse transcriptase inhibitors include adefovir (Preveon® an adenine nucleotide analog from Gilead Sciences), abacavir (1592U89 available from Glaxo Wellcome), and lubocavir (a guanosine analog available from Bristol Meyers-Squibb). Non-nucleoside reverse transcription inhibitors include nevirapine (Viramune™ available from Boehringer Ingelheim Pharmaceuticals, Inc.), delaviridine (Rescriptor® available from Pharmacia & Upjohn) and efavirenz (available as Sustiva®, from DuPont Merck)

Currently, antiviral therapy requires doses of ddI at 200 mg per day BID for an adult human, or in the alternative 400 mg once a day (QD). Similar dosages may be used in the present invention. However, use of the combination drugs may increase the effectiveness of these nucleoside phosphate analogs so that they can be used at lower dosages or less frequently. In combination with hydroxyurea, the presently preferred range for ddI is 100-300 mg twice a day (BID) or 400 mg once a day (QD), assuming an adult weighing 70 kg. When d4T is used with either hydroxyurea or a combination of hydroxyurea and ddI, the preferred range is 40 mg BID.

Of the potential protease inhibitors, compounds such as hydroxyethylamine derivatives, hydroxyethylene derivatives, (hydroxyethyl)urea derivatives, norstantine derivatives, symmetric dihydroxyethylene derivatives, and other dihydroxyethylene derivatives have been suggested, along with protease inhibitors containing the

dihydroxyethylene transition state isostere and its derivatives having various novel and high-affinity ligands at the P_2 position, including 3-tetrahydrofuran and pyran urethanes, cyclic sulfolanes and tetrahydrofuranylglucines, as well as the P_3 position, including pyrazine amides. In addition, constrained "reduced amide"-type inhibitors have been constructed in which three amino acid residues of the polypeptide chain were locked into a γ–turn conformation and designated γ-turn mimetics. Other alternatives include penicillin-derived compounds and non-peptide cyclic ureas. At present, the inventors have no basis for distinguishing among the many potential protease inhibitors that may be used in combination with HU and a reverse transcriptase inhibitor.

The protease inhibitors used in the present invention include Indinavir sulfate, (available as Crixivan™ capsules from Merck & Co., Inc, West Point, PA.), saquinavir (Invirase® and Fortovase® available from Hoffman-LaRoche), ritonavir (Norvir® available from Abott Laboratories) ABT-378 (available from Abott Laboratories), Nelfinavir (Viracept®), and GW141 (available from Glaxo Wellcome/Vertex) Tipranavir available from Pharmacia & Upjohn, PD 178390 available from Parke-Davis, BMS-23632 available from Bristol-Myers Squibb, and DMP-450 available from Triangle, JE 2147 available from Agouron.

In addition to reverse transcriptase inhibitors and protease inhibitors, the present invention may utilize integrase inhibitors such as AR177 (Zintenvir® available from Aronex); fusion inhibitors such as pentafuside, (T-20) and cytokine inhibitors (available from Chiron), chemokine inhibitors, and antisense oligonucleotides such as GPI-2A available from Novopharm Biotech, ISIS-13312 available from Isis, and GEM-132 and GEM-92 available from Hybridon.

Suitable human dosages for these compounds can vary widely. However, such dosages can readily be determined by those of skill in the art. For example, dosages to adult humans of from about 0.1 mg to about 1 g or even 10 g are contemplated.

The combination of compounds of the present invention may be administered by any conventional route. Administration may be oral, intravenous, intraperitoneal, intramuscular, subcutaneous, transdermal, transmucosal (e.g., by inhalation or by means of a suppository), or by any other suitable route. Administration orally in a physiologically acceptable buffered solution is preferred. The buffered solution may be used for one

or more members of the combination, while the other member or members may be administered in another form. Typically, the different members of the combination may be administered on different schedules.

The particular dosage, toxicity, and mechanism for delivery of the individual of drugs of the present invention are either already known, or can be readily determined by conventional empirical techniques, as can dosages for the combination. The combination may result in the ability to use lower amounts of one or more of the constituents. This aspect of the invention may be particularly valuable with respect to the protease inhibitors, which generally are poorly soluble in water and have poor bioavailability. The present invention may address this problem in part by allowing lower dosages. One of ordinary skill in the art will recognize that different dosages and intervals may be appropriate. In the case of children, dosages would tend to be lower due to their smaller mass. This combination would be expected to be particularly useful for children, as the HIV infection tends to result in more brain damage in children, and this combination has good effectiveness in crossing the blood-brain barrier.

The present invention may be used before and after acute infection, before seroconversion, and after seroconversion. In particular, the data presented herein demonstrates an early treatment of the infection that may result in a profound modification of the natural evolution of the HIV-1 infection. Further, the hydroxyurea-containing combinations of the present inventions might be administered prophylactically to high-risk individuals, or to individuals failing protease inhibitor therapy.

In addition, the present invention allows for variation in the mode of treatment over time. Since there are multiple sources of new viral particles being produced during the course of the disease, different drug combinations may used to control them. The amount of virus in the blood together with the presence or absence of other markers such as the numbers of various classes of immune system cells and their response to antigens, can be used as an indicator of which type of combination is most advantageously used. The protease inhibitors are known to be most useful in certain types of activated T-cells that are actively producing virus, often in enormous amounts. They are less effective in quiescent cells. Where very high viral loads in the blood indicate that certain types of activated T-cells are producing large amounts of viral particles, a combination including a protease inhibitor may be indicated. Such a combination may also

advantageously include hydroxyurea and one or more reverse transcriptase inhibitors, integrase inhibitors, fusion inhibitors and cytokine inhibitors. Particularly preferred are the reverse transcriptase inhibitors including AZT, 3TC, ddC, ddI, d4T, abacivir, adefovir, nevirapine, delviridine, efavirenz, and mixtures thereof. Of these, ddI and d4T and mixtures thereof are most preferred.

The protease inhibitor-containing combinations might be reserved for the initial phase of therapy until the viral load is reduced in the plasma (less than 500 copies per milliliter) for longer than 2 months. At this point, the protease inhibitors have very likely reached all the virus producing cells in the reservoirs they can access and have blocked active replication of the virus. Where viral loads in the blood are lower, the activated cells are producing less virus, and the role of the quiescent cell begins to predominate. Then a combination targeting cells such as quiescent lymphocytes and macrophages is indicated. Generally, where the level of virus in the blood is about 50,000 copies per mL or less, combinations including hydroxyurea and one or more reverse transcriptase inhibitors are preferred. Where virus in the blood has reached about 500 copies per mL, and especially when it is 200 copies per mL or less, such combinations are even more preferred. Particularly preferred for use with hydroxyurea are the reverse transcriptase inhibitors including AZT, 3TC, ddC, ddI, d4T, abacivir, adefovir, nevirapine, delviridine, efavirenz, and mixtures thereof. Of these, ddI and d4T and mixtures thereof are most preferred.

Since the HU/reverse transcriptase inhibitor combination is well tolerated, it can be used for long-term therapy. Where the initial viral load in the blood is 50,000-100,000 or less, or when the patient is failing other therapies, the hydroxyurea/reverse transcriptase inhibitor combinations without the protease inhibitor can be used. Where the viral load in the blood is no more than about 20,000 copies per mL, it is preferred that the treatment be continued for at least about 12 to 15 months. Depending on the status of the patient, the time of the treatment can be from several months to lifelong.

Another mode of treatment would be to deliberately activate certain types of quiescent cells during intensive triple combination therapy. Certain quiescent cells do not express HIV-1 proteins, and act as particularly stubborn reservoirs for the virus. In these cells, the HIV-1 DNA is integrated and both gene expression and virus production is only

activated together with the activation of the cells. The cells may remain dormant for years before they spontaneously activate, and begin producing virus particles with the same ferocious reproductive rate and mutation rate as the original, acute infection. None of the presently known drugs can eliminate integrated viral DNA. This difficulty could be overcome if these cells were activated during effective combination therapy. The cells could be activated by vaccination against any of a number of diseases known to activate such cells, including, for example, HIV-1, Hepatitis B, Influenza, and Polio vaccination. HIV-1 genetic immunization is preferred, as disclosed in USSN 60/604,627, filed February 21, 1996. Such activation should preferably take place after the elimination of active virus production (that is, after the patient's viral load is undetectable for at least 2 months). Repeated activation would be helpful to ensure that all quiescent cells harboring HIV-1 DNA had been activated.

One factor influencing the outcome of such a strategy is likely to be the status of the patient's immune system. Although viral load is an important and useful indicator of disease progress, the inventors have found that it is not the only indicator of disease progress, and it can be misleading. The inventors have found, for example, that a combination therapy which allows continuing low levels of viremia to remain in the individual's system can be more effective in inhibiting viral rebound than a drug combination that reduces viral load more quickly and to lower levels.

Examination of changes in the behavior and relative numbers of different classes of immune system cells affected by HIV infection can also provide additional useful information about an individual's immune status. For example, an increase in the number of CD4-T-lymphocytes is considered some evidence of immune system recovery, but an increase in the percentage of "naive" lymphocytes, that is, lymphocytes which have not been exposed to the virus, and which are able to recognize new antigens, together with functional competence of CD4 T-lymphocytes to elicit a vigorous HIV specific immune response from helper T- cells appears to be a more accurate predictor of whether an individual is likely to experience viral rebound if drug therapy is discontinued.

Where the immune system is reconstituted in infected individuals, a vigorous HIV-specific response from CD4+ helper cells can be detected in the peripheral blood. However, $CD4^+$helper T cells are crippled very early in the course of HIV disease. See Vigorous HIV-1-Specific $CD4^+$T Cell

responses Associated with Control of Viremia, Rosenberg, et al., Science Magazine 278:5342 (Nov. 1977) 1447-1450. The patient BM who had stopped therapy without rebound has a high level of HIV-specific $CD4^+$ helper activity, unlike both untreated patients and those treated with conventional therapies including non-hydroxyurea drug combinations containing protease inhibitors. Science Magazine 278:5342 (1977) 1447-1450. The patient's immune system may now play a pivotal role in reducing the replication competence of the HIV viral population. This situation may have been induced by the drug combination, using autovaccination, or prime-and-boost stimulation of the immune system.

Classic vaccination techniques often utilize a prime-and-boost method of enabling the immune system to successfully recognize and control pathogens. That is, the immune system receives a stimulus, has time to recover, and then is exposed again. This sequence may occur several times, depending on the disease, and may be repeated over the course of a lifetime. The experience of BM resembles such a course of treatment. See Fig. 4.

In this case, the primary exposure would have been the initial viral infection. Drug therapy allowed the discontinuation between the primary infection and the boost. Further drug therapy may have allowed another rest interval before a second stimulation, due to the hepatitis infections. Support for this view is found in Example 3, where four out of five patients treated with hydroxyurea-based triple combination therapy tested negative for HIV competent virus using the standard test, and two tested negative when the test sensitivity was increased tenfold. Significantly, the only patient to test positive had no history of either vaccination or exposure to hepatitis in some form.

It may be possible, therefore, to treat patients in the primary infection stage, that is, patients undergoing the process of seroconversion by administering an effective viral control drug combination until the level of virus in the blood is between about 50± 1 log copies per mL or less, stop therapy and allow rebound to about 5,000 ± 1 log to 50 ± 1 log copies per mL to occur, then restart therapy for at least three weeks, or until replication competent virus is no longer detectable in the body. In the alternative, where the viral load in plasma is less than 500 ± 1 log copies per mL, the patient may be vaccinated without stopping therapy. To achieve this result, the drug therapy must be capable of inhibiting replication of HIV in the

presence of low levels of virus replication. The only known combinations capable of doing this are those containing hydroxyurea. See Examples 3, 8 and 9.

Use of hydroxyurea may be essential to the use of this prime-and-boost technique because of its function. Hydroxyurea modulates the immune system. That is, it reduces the rate of activation of immune system cells somewhat, or has a mild cytostatic effect. When HIV infects CD4+ helper cells, the CD8 (killer) cells attack. Normally this would lead to control of a pathogen. But this activity does not eliminate the HIV infection, so the CD8 cells kill the CD4+ helper cells, and more CD8 cells are activated, which results in the killing of more CD4+ helper cells until the net result is a loss of the $CD4^+$ helper cells and exhaustion of CD8 cells. This overexpansion of the immune system is ineffective to control the virus, which simply keeps replicating.

A mechanism to control the virus is desirable, but the immune system must be kept from overexpanding. Hydroxyurea combination therapy is believed to inhibit HIV replication and also down-modulate CD8 activation. The result is CD4+ helper cell population recovery, and the CD8 cells are not exhausted.

The following Examples are presented for the purpose of illustrating the practice of the present invention. They do not limit the invention, or the claims which follow.

Examples

A key step of HIV-1 infection of lymphocytes is the conversion of the viral RNA genome into double-stranded DNA by the action of HIV-1 RT. Viral DNA synthesis differs in different states of infected lymphocytes. In quiescent cells, viral DNA synthesis can be initiated as efficiently as in activated cells. However, in contrast to the activated cells, DNA synthesis in quiescent lymphocytes may terminate prematurely (J. A. Zack, et al., *Cell* **61**:213 (1990) ; J. A. Zack, et al., *Virology* **66**:1717 (1992)) producing no HIV-1 progeny (Zack, et al, supra; M. Stevenson, et al., *EMBO J.* **9**:1551 (1990); M. I. Bukrinsky, et al., *Science* **254**:423 (1991)). This process results in a pool of unintegrated viral DNA (Stevenson, et al., *supra*; Bukrinsky, et al., *supra*), which can remain latent in both *in vitro* infected quiescent peripheral blood lymphocytes and *in vivo* infected resting peripheral blood lymphocytes (Zack, et al., *supra*, 1990 & 1991; Stevenson, et al., *supra*; Bukrinsky, et al., *supra*). Activation of these cells can rescue

HIV-1 DNA, leading to integration and production of viral progeny (*Id.*). Incomplete viral DNA has also been found associated with HIV-1 mature infectious particles, but the biological role of this DNA is unclear (F. Lori, et al., *J. Virol.* **66**:5067 (1992) ; D. Trono *ibid.* **66**:4893 (1992)).

Example 1 illustrates the various methods that can be used to quantitate the replication of the HIV-1. A variety of different tests with different sensitivities are currently in use, particularly since researchers have found that older screening methods with a sensitivity of < 400 copies per milliliter plasma are simply not sensitive enough to tell whether a dangerous infection continues to exist in the individual. It has also been demonstrated that lymphoid tissues are the major reservoirs of HIV-1,(See Pantaleo, G., Graziosi, C., Demarest, J.F., Butini. L., Montroni, M., Fox, C.H., Orenstein, J.M., Kotler D.P., Fauci, A.S. *HIV infection is active and progressive in lymphoid tissue during the clinically latent stage of disease.* Nature **362**(6418): 355-358 (1993) therefore, new detection methods for HIV-1 RNA and DNA have been developed and applied to the lymph tissues. Of the newer methods, the most sensitive used herein is the nested PCR assay detecting HIV-1 DNA (sensitivity: one copy of virus per sample) applied to one half of a lymph node. Another new method is the in situ hybridization detection of HIV-1 RNA, (See Fox C.H., Cottler-Fox, M. *In situ hybridization for the detection of HIV RNA in cells and tissues.* Current Protocols in Immunology (Coligan, J., Kruisbeek, A., Margulies, D., Shevack E., Strober, W. eds), Wiley, NY, 1993 ; and Fox C.H., Cottler-Fox, M. *In situ hybridization in HIV research.* J. Microscop. Tech. Res. **25**:78-84, 1993.) can be applied to the other half of the lymph node. A more typical sample size as reported in the current literature would be obtained via biopsy of the lymph node rather than its complete surgical removal.

Example 1

<u>HIV Replication</u>

Inguinal lymph nodes were surgically removed and cut in half along a longitudinal axis. One part was fixed in formalin for in situ hybridization and the other part was frozen in liquid nitrogen. The frozen tissue was homogenized and its DNA was extracted. HIV-1 DNA was amplified by a highly sensitive polymerase chain reaction (PCR assay), described in detail in Methods in Molecular Biology, Vol. 15: PCR Protocols .

PCR SK primers SK38 and SK39 are available from Perkin-Elmer, Norwalk, CT. The PCR-reaction mixture contained the following: 500 ng of genomic DNA, 0.2 mM of each primer, 100 FM of each nucleoside triphosphate, 1.5 mM $MgCl_2$, 20 mM Tris-HCl (pH 8.4), 50 mM KCl and 1 units of Taq DNA polymerase (Boheringher Manheim Corporation, Indianapolis, IN) in a final volume of 100 Fl. The cycle conditions were 95 °C for 3 minutes, 50 times (94 °C for 1.30 min, 56 °C for 1.00 min and 72 °C for 1.00 min) and 72 °C for 10 minutes.

The following PCR RT primers were designed and used by the inventors: sense-primer RT-F1 (5-GGACCTACACCTGTCAACAT-3, nucleotides 127 to 146 of HXB2 pol gene) and antisense-primer RT-R8 (5-CATTTATCAGGATGGAGTTCATA-3, nucleotides 886 to 908 of HXB2 pol gene)

The PCR-reaction mixture contained the following: 500 ng of genomic DNA, 0.2 FM of each primer, 100 FM of each nucleoside triphosphate, 2 mM $MgCl_2$, 20 mM Tris-HCl (pH 8.4), 50 mM KCl and 1 units of Taq DNA polymerase (Boheringher Manheim Corporation, Indianapolis, IN) in a final volume of 100 Fl. The cycle conditions were 95 EC for 3 minutes, 50 times (94 EC for 1.30 min, 56 EC for 1.30 min and 72 EC for 1.30 min) and 72 EC for 10 minutes.

Hybridization primers:

RT - F7 GGATGGAAAGGATCACCAGC

RT - R6 TACTAGGTATGGTAAATGCAGT

NESTED-PCR (For Increased Sensitivity)

Sense-primer RT-F5 (5-CAGGAATGGATGGCCCAAAAGT-3, nucleotides 233 to 254 of HXB2 pol gene) , antisense-primer RT-R12 (5-TTCATAACCCATCCAAAG-3, nucleotides 874 to 891 of HXB2 pol gene).

PCR conditions were 1 Fl from the first PCR reaction, 0.4 FM of each primer, 200 FM of each nucleoside triphosphate, 1.5 mM $MgCl_2$, 10 mM Tris-HCl , 50 mM KCl, and 1 unit of Taq DNA polymerase (Boheringher), in a final volume of 50 Fl.

The cycle conditions were 95EC for 3 minutes, 45 times (94EC for 30 sec., 55 EC for 30 sec. and 72 EC for 30 sec.), and 72 EC for 10 minutes.

The DNA from PCR reaction was separated on an agarose gel and visualized by Ethidium Bromide staining. Polaroid pictures were taken. To

increase the sensitivity at least 100 fold, the DNA was blotted to nitrocellulose paper and hybridized with a fluorescent- labeled oligonucleotide according to the manufacturer protocol (ECL 3-oligolabelling and detection systems. Amersham Life Science, Little Chalfont, England).

Primer F1 was previously described by Xiping W, Ghosh S, Taylor M, Johnson V, Emini E, Deutusch P, Lifson J, Bonhoeffer S, Nowak M, Hahn B, Saag M, Shaw G. *Viral dynamics in human immunodeficiency virus type 1 infection.* Nature 1995;373:117-122 ; Primer F5 was described by Saag, M.S., Emini, E.A., Laskin, O.L., Douglas, J., Lapidus, W.I., Schleif, W.A., Whitley, R.J., Hildebrand, C., Byrnes, V.W., Kappes, J.C., Anderson, K., Massari, F., Shaw, G., and the L-697 working group. *A short-term clinical evaluation of L-697,661, a non-nucleoside inhibitor of HIV-1 reverse transcriptase.* L-697,661 Working Group. N. Engl. J. Med. 1993;329:1065-72.

__Genomic DNA extraction from lymph nodes__

Extraction of DNA from whole tissue was done by using a DNA extraction kit available from Stratagene, La Jolla, CA, according to the manufacturer's instructions. The only modification was that the frozen lymph nodes were first ground to a powder in a porcelain mortar under liquid nitrogen, and then the powder was transferred into a Wheaton Potter-Elvehjem tissue grinder and homogenized in a lysis buffer. Incubation with protonase was done at 37 °C overnight.

__Viral load quantitation by NASBA™ in semen__

Quantitation of HIV-1 RNA in semen was performed by using a NASBA™ HIV-1 RNA QT kit available from Organon Teknika, Netherlands, according the manufacturer's protocol. Briefly, 200 µl of semen were mixed with 1.8 ml of lysis buffer and frozen until use. Nucleic acids were extracted using a guanidine thiocyanate-silica based method (Boom, R., Sol, C.J.A., Salimans, M.M.M., Jansen, C.L., Wertheim-van Dillen, P.M.E., van der Noordaa, J. (1990) *A rapid and simple method for purification of nucleic acids.* J. Clin. Microbiol. **28:**495-503 and van Gemen, B., Kievets, T., Schukkink, R., van Strijp, D., Malek, L.T., Sooknanan, R., Huisman, H.G., Lens, P. (1993) *Quantitation of HIV-1 RNA in plasma using NASBA™ during HIV-1 primary infection.* J. Virol. Meth. **43:** 177-188.) Amplification of

the target HIV-1 RNA by NASBA™ was performed with primers specific for the *gag* region of the HIV-1 genome (Kievits, T., van Gemen, B., van Strijp, D., Schukkink, R., Dircks, M., Adriaanse, H., Malek, L., Sooknanan, R., Lens, P (1990) *NASBA™ isothermal enzymatic in vitro nucleic acid amplification optimized for the diagnosis of HIV-1 infection.* J. Virol. Meth. **35**: 273-286 , and van Gemen, B., van Beuningen, R., Nabbe, A., van Strijp, D., Jurriaans, S., Lens, P., Kievits, T. (1994) *A one-tube quantitative HIV-1 RNA NASBA nucleic acid amplification assay using electrochemiluminescent (ECL) labeled probes.* J. Virol. Meth. **49**: 157-168.

Example 2

Six individuals were treated with the combination of hydroxyurea, a nucleoside analog, and a protease inhibitor. The general course of treatment was HU, 5-8 mg/kg TID; ddI, 200 mg BID, Indinavir 800mg/TID. Three of them were treated within 4-7 weeks following primary infection and before seroconversion, that is, when the Western Blot was not completely positive. Figs. 1-3 contain the corresponding Western Blot information for these patients. Three patients were treated from <1 year to >5 years after seroconversion. In all the individuals the levels of plasma viremia became undetectable within 3-25 weeks after treatment. All the data is shown below in Tables 1-6, each of which is further identified by a two-letter code.

Three individuals began treatment within 14 to 31 days following the onset of symptoms (DFOS) of a primary HIV-1 infection and before seroconversion. In all of these individuals, the levels of plasma viremia became undetectable within 73 to 136 DFOS (with a drop of viremia between 2.7 to 3.4 logs) and remained undetectable during the course of treatment. None of these patients fully seroconverted during the course of this study, despite a documented exposure to HIV-1 ranging between 163 and 236 days. Their Western Blot patterns remained almost unaltered during the course of the observation. See Tables 1-3, for results of BM, FC, and SH, and Figs. 1-3 for the original and subsequent Western Blot results. Moreover, a significant, sharp increase of the CD4/CD8 ratio and CD4 count was observed in all three patients. Lymph nodes were collected from these patients at different time points to detect HIV-1 RNA by in situ hybridization.

The first node of FC was analyzed 8 weeks after beginning treatment, while the virus was still detectable in the plasma, and HIV-1 RNA was mainly associated to the follicular dendritic cells. At this time, the CD4 count and CD4/CD8 ratio was normal. Later, when HIV-1 was no longer detectable in the plasma, another inguinal lymph node was obtained and analyzed as before. No HIV-1 RNA was detected at that time. Again, DNA was extracted from the half of the lymph node which was frozen and tested by PCR analysis using 2 different primers and also a nested primer (sensitivity of this test is to one copy of viral DNA per sample). The PCR was positive, indicating that FC had at least one copy of HIV-1 DNA in the lymph node. See Table 1.

In patient SH, HIV-1 became undetectable in the plasma at 105 days after treatment had begun, with a decrease in viremia of 3.2 log from the baseline. The CD4 counts and CD4/CD8 ratios increased promptly after treatment began (from 0.33 to 0.95 in 33 days). In two consecutive analyses, no RNA was detected in the lymph nodes, but at least 1 copy of viral DNA was detected at 176 days from the onset of symptoms and 145 days from the start of treatment. No significant changes were observed in the Western blot profile of SH during the course of the follow-up. See Table 2.

The third patient (BM, see Table 3) was treated starting 7 weeks after the probable date of infection. Between 19 and 22 days after starting the therapy, he interrupted the treatment for three days, concomitantly with an episode of orchitis. A rebound of plasma viremia was monitored immediately after the three day suspension. Therapy was started again, and at about week 5 after initiating treatment, HIV-1 was undetectable in the plasma. At week 16, an inguinal lymph node was analyzed and 2 RNA producing cells were found out of 44 million cells screened. At week 17, treatment was again interrupted, this time due to an episode of acute Hepatitis A. Despite the massive immune stimulation due to this concomitant viral infection, this individual did not show any sign of viral rebound during the following two weeks, although a positive value was found but could not be confirmed. The same week, BM again started taking the therapy. After an additional 4-5 weeks of therapy taken at irregular intervals, the patient discontinued treatment altogether. Another lymph node was obtained 18 days following final suspension of therapy, and 2 RNA producing cells were found out of a total of 44 million cells

screened. No DNA could be detected in this lymph node, even after repeated nested PCR analysis. The plasma tested positive for RNA 40 days after treatment suspension at very low levels, but this positivity could not be confirmed. During all the course of the follow-up, cell counts did not significantly change, and the Western blot profile remained practically unchanged. Semen of patients FC, SH and BM was tested at 141, 176, and 214 days from onset of symptoms, respectively, and HIV-1 RNA was undetectable by NASBA (sensitivity <400 copies/ml). Similarly, the semen of the other patients, when tested, showed negative results.

The viral load variations in the plasma and the changes in the CD4 and CD8 absolute/relative counts of the three patients who were treated after seroconversion did not differ significantly from those who were treated before seroconversion. Patients TD and LF showed sharp increases in CD4 counts back to normal levels and their CD4/CD8 ratios now range between 1.1 and 1.4. See Tables 4 and 5. The one patient who had the longest (>5 years) infection before therapy and started with the lowest CD4 count (330), LJ, showed marked improvement also, but progress was slower. This patient became virus negative in the plasma only after 25 weeks of treatment, and the increase of the CD4/CD8 ratio was slower and less significant than in the other individuals. The CD4/CD8 ratio remained at about 0.4 to the end of this study. See Table 6.

The patient who had been seropositive for the longest period of time, LJ, (See Table 6) had low but detectable levels of HIV-1 RNA and proteins in the lymph node after 27 weeks of treatment.

Patient TD had been seroconverted for approximately 6 months before treatment had begun. Patient TD currently shows no traces of viral DNA or RNA in the sperm, serum, or lymph nodes. This patient has had a history of hepatitis infection in March, 1996.

These data indicate that the combination of hydroxyurea, ddI and a protease inhibitor present a potent new combination that can rapidly clear the virus from plasma and lymph nodes, and inhibit viral rebound after cessation of treatment. Further, this combination blocks HIV-1 replication in the lymphoid system and, at least in one case, shows hope for HIV-1 eradication.

In addition, these data indicate that HIV-1 infection is treatable as an emergency disease. Patients should be tested not for seroconversion, but for free virus particles in the blood if there are any symptoms or suspicion

of infection, and treated immediately with the combination therapy before seroconversion takes place. The present invention will eliminate the free virus, block new infections, restore the immune system, and may eliminate virus integration in millions of cells. This method would also be economical, as treatment would be begun earlier and be of shorter duration than treatment for people with chronic infections. The present results show that the patients treated early restored the normal lymphocyte status in short periods of time (see CD4+cells and CD4/CD8 ratio). However, the patient that had been infected for 5-9 years (LJ, see Table 6) could not as rapidly restore the lymphocyte status even after 9 months of therapy, even though virus production was completely blocked. This demonstrates that the in vivo clearance rate cannot be generalized for all treatments that apparently reduce the presence of virus in plasma. Further, the same patient, unlike the patients with fresh infections, did not demonstrate restoration of the T-cell repertoire (CD4 counts and CD4/CD8 ratios) to the normal levels. This result indicates that late in the infections, T-cells do not turn over at the same rate as they did earlier, and regeneration of T-cells may be impaired. It further suggests early treatment.

The present results also indicate a method of eliminating quiescent cells, that is, cells that have integrated viral DNA, but do not currently express the genes or produce virus. HIV-1 DNA was measured in the lymphocytes of the patients with a highly sensitive nested PCR able to detect as little as 1 copy of viral DNA. Two patients in this group had no detectable HIV-1 DNA in the lymphoid organ and 3 had detectable DNA. Other investigators have also reported (in all cases) detectable DNA in the lymphoid organs, even in the absence of virus producing cells. (Markovitz, Retrovirus Conference, 1997). The present inventors are not aware of any other patients other than the two in the present study (TD, BM) who have undetectable viral DNA in the lymphoid organs. The only common feature of these patients is that both experienced hepatitis infection. BM had Hepatitis A and TD had hepatitis B. Both infections are characterized by activation of cells which can harbor HIV-1 DNA. After activation, these cells can produce viral particles that will be mainly defective in the presence of protease inhibitors. HU and ddI will work at the early phase, inhibiting reverse transcription with two different mechanisms, consequently blocking both new infection and new DNA integration.

The present results also show that early treatment of individuals (BM, SH, FC and TD, LF) infected by HIV-1 led to a profound modification of the natural evolution of HIV-1 infection.

First, HIV-1 became and remained undetectable in the plasma. The implication of this result is highly relevant. In fact, only 8% of individuals with less than 4,350 copies of RNA per milliliter of blood plasma soon after infection developed AIDS 5 years after infection, whereas 62% of those with values greater than 36,270 progressed to AIDS (Mellors, J. W. et al., Science 272(5265): 1167-1170, 1996). In the ACTG 175 study, a decrease of 1.0 log in the concentration of HIV-1 RNA from the baseline after therapy with nucleoside analogues in patients with CD4 counts between 200 and 500 per cubic millimeter was associated significantly with a 65% reduction in the risk of AIDS or death (N.Engl. J. Med. 1996 Oct 10:335(15):1091-8). All the patients analyzed here had high levels of viral replication (between 89,390 and 487,955 copies/mL) before the treatment and this load was decreased between 2.7 and 3.4 logs, that is, below 200 copies/mL.

Second, also in the lymph node compartment, which has been described as the major reservoir of the virus, only traces of HIV-1 RNA and/or DNA could be inconstantly detected. In particular, follicular dendritic cell-associated HIV-1 found in patient FC 57 days following treatment rapidly disappeared 70 days later, indicating the rapid clearance of follicular dendritic cell-associated HIV-1 following this therapy.

Third, CD4 counts increased promptly to normal levels and CD4/CD8 ratios were normalized in patients LF, TD, FC, SH, BM, whereas these values typically fail to increase to normal.

Fourth, lack of a full seroconversion in 3 of these patients treated prior to seroconversion suggests that the replication of the virus has at least been reduced to a minimum. Of particular interest is the observation that all of the above considerations hold true even after the treatment has been suspended in one of the patients. Despite a possible smoldering expression of viral RNA, DNA was repeatedly undetectable in the lymph nodes, even with a methodology able to detect a single copy of viral DNA.

Fifth, we have recently shown on an animal model (unpublished) that an early treatment with ddI and ddI with hydroxy urea, although unable to prevent the infection of pigtail macaques by a lethal dose of SIV, reduced the viral load and rescued the animals from death. This also

demonstrates that early, effective treatment can completely change the course of retrovirus infection.

The combination of hydroxyurea, 2',3'-dideoxyinosine (ddI) and Indinavir during the acute primary phase of infection resulted in a very potent, long lasting block of HIV-1 replication in the blood, lymph nodes and semen and in the restoration of the immune system. In one patient, the treatment was suspended without substantial viral rebound or seroconversion.

Example 3

Samples from five patients treated with hydroxyurea, ddI and a protease inhibitor, as well as variable histories of immune system stimulation by way of vaccination or natural hepatits infection were analyzed according to a new and highly sensitive method for hidden HIV infection. All patients were found to have extraordinarily low levels of latent HIV, and a history of vaccination or infection by Hepatitis A or B was associated with a further decrease in HIV found in quiescent cell reservoirs.

The test method is described in Identification of a Reservoir for HIV-1 in Patients on Highly Active Antiretroviral Therapy, Finzi et al., Science Magazine 278 (5341):1295-1300. This is a test for replication-competent virus that may persist in the resting CD4+T cells of patients who have no evidence of active virus replication. In tests conducted on patients who had been on regimens including three drugs selected from the groups nucleoside analogs (AZT, d4T and 3TC), nonnucleoside reverse transcriptase inhibitors (nevirapine) and protease inhibitors (ritonavir, saquinavir, indinavir and nelfinavir), who had good response to treatment, all patients were found to have a low frequency of CD4+ T cells harboring replication competent latent HIV-1, on the order of 0.2 to 16.4 per million cells.

A patient treated with hydroxyurea, ddI and Indinavir who had been exposed to Hepatitis A and who had discontinued treatment for about a year (patient BM, above) tested negative for the standard test. The test sensitivity was increased by a factor of 10, and the patient registered as positive on the more sensitive test.

A patient treated with hydroxyurea, ddI and Indinavir that switched a year later to hydroxyurea, ddI and nelfinavir (SH above, due to tolerance problems) was deliberately vaccinated with Hepatitis A, and tested. This

patient was negative on the standard test and positive on the more sensitive test.

A patient treated with hydroxyurea, ddI and Indinavir that later switched to nelfinavir was not vaccinated, and scored positive on the standard test. This is the only one who was positive on the first test, and the only one who was not either vaccinated or naturally exposed to hepatitis.

Two patients began with hydroxyurea, ddI, nevirapine and nelfinavir, and both switched after one month from nevirapine to delaviridine. One was not vaccinated, but tested positive for Hepatitis B antibodies. The other was not vaccinated, but tested positive for Hepatitis A antibodies. Both were negative on the standard test.

In this case, four our of five patients tested as having no replication competent virus. When the test sensitivity was increased by a factor of 10, two patients were found to have less than 1 cell per 10 million having replication competent virus.

TABLE 1 - VIRAL LOAD, LYMPHOCYTE AND SEROCONVERSION ANALYSES FOR PATIENT FC

Infected: End July beginning of August according to Heiko
3 day break in therapy: Sept 15 - 18, 1996

Therapy begun 2 Sept. 96
HU 400 mg TID
ddI 200 mg BID
INDINAVIR 800 mg TID

VIRAL LOAD IN THE BLOOD			
D/M/Y	bDNA/PCR	PCR/RIGHT	
23.8.96	800,000		
26.8.96	1,280,000		
2.9.96	785,000	487955	start 2-9
9.9.96	37,000	22497	stop 15-18
20.9.96	21,000	13929	
27.9.96	500	5919	
29.10.96	500	673	
18.11.96	400	<200	
23.12.96	400	<200	
6.1.97		<200	
14.01.97	500		
29.1.97		<200	

VIRAL LOAD IN THE LYMPH NODE				
D/M/Y				
p24 antigen expression		HIV-RNA in situ		HIV DNA PCR
29.10.96				
FDC	Cells	FDC	Cells	
-	-	+	(few) +	Not detected
07.01.97				
-	-	-	-	positive

VIRAL LOAD
SEMEN
07.01.97
<400 copies/ml

LYMPHOCYTE POPULATION ANALYSIS								
D/M/Y	Lymph.	B.Ly	T-Ly	CD4	CD4 %	CD8	CD8 %	Ratio
26.10.96	2,543	200	2,010	1,220	48	970	38	1.26
18.8.96	1,336	110	900	380	28	680	51	0.56
23.8.96	1,548	80	1,020	330	21	850	55	0.39
27.8.96	1,974	99	1,210	474	24	1,046	53	0.45
2.9.96	3,256	130	2,583	684	21	2,084	64	0.33
9.9.96	2,415	242	1,771	652	27	1,232	51	0.53
20.9.96	1,546	216	1,199	680	44	526	34	1.29
18.11.96	2,149	279	1,624	946	44	688	32	1.38
06.01.97	1,480	252	1,139	696	47	459	31	1.52
29.01.98	2,416	314	1,705	821	34	870	36	0.94

SEROCONVERSION BY WESTERN BLOT TEST									
Date	HIV 1+2	gp160	gp120	p65	p55	gp41/43	p32	p24	p18
19.08.96	±	±	-	-	-	-	-	±	-
23.08.96	±	+	±	-	-	-	-	±	±
02.09.96	±	+	±	-	-	-	-	+	+
09.09.96	+	+	±	-	-	-	-	±	±
27.09.96	+	+	±	±	-	-	-	+	+
23.12.96	+	+	±	±	±	±	±	±	±

Table Legend:

1. Each table summarizes viral load, lymphocyte and, where applicable, seroconversion analyses for a single patient over time.
2. Dates are listed as day/month/year "D/M/Y".
3. Viral load was measured on the dates indicated by a contract laboratory ("bDNA/PCR") or by the inventor's laboratory ("PCR/RIGHT").
4. Results for tests for viral load in the lymph nodes were recorded as follows:

"p24 antigen expression": measured by antibodies against HIV protein p24 applied to a thin layer of lymnph node tissue.

"HIV-RNA in situ": measured by nucleotide binding to lymph node tissue.

"HIV DNA PCR": standard test for viral load. "Not detected" means not tested on that date.

"FDC": Follicular Dendritic Cells

"Cells": Lymph node cells generally.

5. Lymphocyte population analysis shows the complement of the patient's lymphocytes at the given dates.

"Lymph.": Total number of lymphocytes.

"B.Ly": Total number of B-lymphocytes.

"T-Ly": Total number of T-Lymphocytes.

"CD4" Total number of CD4+ lymphocytes.

"CD4%": The percentage of total lymphocytes that are CD4+ lymphocytes.

"CD8": Total number of CD8+ lymphocytes.

"CD8%": The percentage of total lymphocytes that are CD8+ lymphocytes.

"Ratio": The CD4+/CD8+ ratio.

6. Seroconversion was shown by Western Blot analysis. The patient's blood was screened for antibodies to the listed HIV proteins at the given dates.

TABLE 2 - VIRAL LOAD, LYMPHOCYTE AND SEROCONVERSION ANALYSIS OF PATIENT SH

Infection: The patient says he was infected June 96
He went to hospital 15 July 96 with high fever

Therapy begun 27 July, 96

HU	400 mg TID
ddI	200 mg BID
INDINAVIR	800 mg TID

VIRAL LOAD IN THE BLOOD		
D/M/Y	bDNA/PCR	PCR/RIGHT
19.7.96	199000	
22.7.96	192000	319146
26.7.96	3260000?	871602
31.7.96	82310	143435
20.8.96		3100
23.9.96	500	918
25.10.96	500	266
28.11.96	500	<200
12.12.96		<200
08.01.97	500	
29.1.97		<200

VIRAL LOAD IN THE LYMPH NODE					
D/M/Y	p24 antigen expression		HIV-RNA in situ		HIV DNA PCR
	FDC	Cells	FDC	Cells	
16.10.96	-	-	-	-	Not detected
07.01.97	-	-	-	-	positive

VIRAL LOAD
SEMEN
07.01.97
<400 copies/ml

LYMPHOCYTE POPULATION ANALYSIS								
D/M/Y	Lymph.	B.Ly	T-Ly	CD4	CD4 %	CD8	CD8 %	Ratio
19.7.96	1,578	170	1,250	520	33	870	55	0.60
26.7.96	1,778	120	1,400	410	23	1,190	67	0.34
2.8.96	2,040	220	1,570	590	29	1,140	56	0.52
20.8.96	1,911	230	1,450	590	31	1,010	53	0.58
17.9.96	2,012	302	1,549	804	40	845	42	0.95
25.10.96	1,816	182	1,412	672	37	708	39	0.95
12.12.96	1,742	244	1,352	645	37	627	36	1.03
14.01.97	2,424	267	1,798	776	32	970	40	0.80
29.01.97	2,133	299	1,527	661	31	832	39	0.79

SEROCONVERSION BY WESTERN BLOT TEST									
D/M/Y	HIV 1+2	gp160	gp120	p65	p55	gp41/43	p32	p24	p18
19.07.96	±	±	-	-	-	-	-	-	-
02.08.96	±	±	-	-	-	-	-	±	-
20.09.96	±	±	-	-	-	-	-	±	-
17.09.96	+	+	±	-	-	-	-	±	-
04.10.96	+	+	±	-	-	-	-	±	-
12.12.96	+	+	±	-	-	-	-	±	±

TABLE 3 - VIRAL LOAD, LYMPHOCYTE AND SEROCONVERSION ANALYSES FOR PATIENT BM

Infection 10 May 96
Testis infection 10 July 96; stop therapy: 12-19 July 1996
Acute hepatitis A infection: stop therapy 26 Oct. 96
Takes pills very irregularly
Stops voluntarily treatment on December 20

Therapy begun 27 June 96

HU	300 mg TID
ddI	200 mg BID
INDINAVIR	800 mg TID

VIRAL LOAD IN THE BLOOD

D/M/Y	bDNA/PCR	PCR/RIGHT	
21.06.96	12620	80041	
9.7.96	<500	1099	stop (12-19)
19.7.96	1134	5356	*rebound*
19.8.96	<500	<200	neg
5.9.96	<500	<200	
1.10.96	<500	<200	stop 26 Oct
29.10.96	<500	<200	
1.11.96		<200	
8.11.96	<400	324; <200	starts 11 Nov
11.12.96	<500	<200;<200	stop 20 Dec
07.01.97	<400	<200;<200	2,5 wks a. stop
16.01.97		279;<200	
27.01.97	<500	221;<200	5 wks a. stop
7.2.97	<500	nd	
14.2.97	700?	nd	

VIRAL LOAD IN THE LYMPH NODE

D/M/Y	p24 antigen expression		HIV-RNA in situ		HIV DNA PCR
	FDC	Cells	FDC	Cells	
16.10.96	-	-	-	2 pos. in 44 million	Not detected
01.7.97	-	-	-	3 pos. in 44 million	negative

VIRAL LOAD SEMEN

07.01.97	<400 copies/ml

LYMPHOCYTE POPULATION ANALYSIS

D/M/Y	Lymph.	B.Ly	T-Ly	CD4	CD4 %	CD8	CD8 %	Ratio
21.6.96	1,408	210	1,020	370	26	710	50	0.52
24.6.96	1,411	230	1,020	410	29	690	49	0.59
26.6.96	1,965	270	1,430	570	29	940	48	0.61
19.7.96	1,643	310	1,130	490	30	620	38	0.79
19.8.96	1,880	280	1,330	660	35	730	39	0.90
1.10.96	1,587	238	1,152	571	36	540	34	1.06
1.11.96	1,907	305	1,426	572	30	801	42	0.71
11.12.96	2,304	392	1,553	991	43	968	42	1.02
7.1.97	2,145	429	1,471	751	35	686	32	1.09
29.1.97	1,961	412	1,302	628	32	588	30	1.07
7.2.97	2,306	369	1,591	738	32	853	37	0.87
14.02.97	2,232	379	1,482	670	30	781	35	0.86

SEROCONVERSION BY WESTERN BLOT TEST

Date	HIV 1+2	gp160	gp120	p65	p55	gp41/43	p32	p24	p18
11.6.96	±	-	-	-	-	-	-	-	-
21.6.96	±	-	-	-	-	-	-	-	-
26.6.96	±	-	-	-	-	-	-	-	-
19.7.96	+	+	-	-	-	-	-	±	-
1.10.96	+	+	-	-	±	-	-	+	-
8.11.96	+	+	±	-	-	-	-	±	±

TABLE 4 - VIRAL LOAD AND LYMPHOCYTE ANALYSES OF PATIENT TD

Infected November 95
Seroconverted December 95
Hepatitis March 96

Therapy begun 29 July 96
HU 300 mg TID
ddI 200 mg BID
INDINAVIR 800 mg TID

VIRAL LOAD IN THE BLOOD		
D/M/Y	bDNA/PCR	PCR/RIGHT
08.03.96	47,940	
13.03.96	<10,000	
25.04.96	39,290	
23.05.96	32,980	
20.06.96	30,090	
18.07.96	45,290	
22.07.96	42,000	
22.07.96	114,000	
01.08.96	48,000	
10.09.96	750	
21.10.96	<500	"neg?"
04.11.96	<500	<200
23.12.96	<500	<200
30.01.97	<400	<200

VIRAL LOAD IN THE LYMPH NODE			
	HIV-RNA in situ		HIV DNA PCR
Cells 07.01.97	FDC	Cells	
-	-	-	negative

VIRAL LOAD SEMEN
07.01.97
<400 copies/ml

LYMPHOCYTE POPULATION ANALYSIS								
Date D/M/Y	Lymph.	B.Ly	T-Ly	CD4	CD4 %	CD8	CD8 %	Ratio
25.04.96	1745	90	1140	490	28	1010	58	0.49
23.05.96	1934	120	1620	620	32	1120	58	0.55
20.06.96	1885	110	1550	550	29	1060	56	0.52
18.07.96	1649	120	1340	480	29	970	59	0.49
10.09.96	2253	203	1794	879	39	1104	49	0.80
23.12.96	1560	156	1213	530	34	640	41	0.83
08.01.96	2504	225	2098	1102	44	1102	44	1.00
30.01.97	2448	269	1928	881	36	979	40	0.90
14.02.97	2545	204	2138	1069	42	1044	41	1.02

TABLE 5 - VIRAL LOAD AND LYMPHOCYTE ANALYSES OF PATIENT LF

Infected May 95?
Naive (never treated before)

Therapy begun 26 June 96

HU	300 mg TID
ddI	200 mg BID
INDINAVIR	800 mg TID

VIRAL LOAD IN THE BLOOD		
D/M/Y	bDNA/PCR	PCR/RIGHT
05.03.96	70,200	
15.03.96	40,950	
04.04.96	14,910	
10.5.96	27,290	
07.06.96	30,820	
12.07.96	< 500	
20.08.96	< 500	
24.09.96	< 500	
10.10.96	< 500	
08.11.96	< 400	
14.12.96	< 500	
7.1.97		<200

VIRAL LOAD IN LYMPH NODE				
D/M/Y				
p24 antigen expression		HIV-RNA in situ		HIV DNA PCR
FDC	Cells	FDC	Cells	
07.01.97				
-	-	-	-	positive

VIRAL LOAD SEMEN
07.01.97
<400 copies/ml

LYMPHOCYTE POPULATION ANALYSIS								
D/M/Y	Lymph.	B.Ly	T-Ly	CD4	CD4 %	CD8	CD8 %	Ratio
06.03.96	2880	194	2095	693	25	1358	49	0.51
22.03.96	2772	168	2193	729	26	1374	49	0.53
10.05.96	3578	250	2650	790	22	1860	52	0.42
07.06.96	2756	190	1930	630	23	1490	54	0.42
12.07.96	2584	260	1880	750	29	1190	46	0.63
26.08.96	2268	249	1701	771	34	998	44	0.77
10.10.96	2508	201	1826	853	34	953	38	0.90
04.12.96	1888	189	1385	642	34	680	36	0.94
07.01.97	2350	235	1833	940	40	870	37	1.08

TABLE 6 - VIRAL LOAD AND LYMPHOCYTE ANALYSES FOR PATIENT LJ

Infected 1987 or 1991
Naive (never treated before)

Therapy begun 28 March 96	
HU	300 mg TID
ddI	200 mg BID
RITONAVIR	600 mg BID

Therapy changed 1 July 96	
HU	300 mg TID
ddI	200 mg BID
INDINAVIR	800 mg TID

VIRAL LOAD IN THE BLOOD	
D/M/Y	PCR/RIGHT
21.2.96	
25.3.96	95229
1.4.96	
23.4.96	757
7.5.96	
30.5.96	836
28.6.96	393
22.7.96	347
6.8.96	
5.9.96	
19.9.96	<200
14.10.96	<200
18.11.96	
4.12.96	<200
18.12.96	
28.1.97	<200

VIRAL LOAD IN THE LYMPH NODE			
D/M/Y			
2.10.96	size: 6.5 x 12.5		2 weeks a. neg
p24 antigen expression		HIV-RNA in situ	
FDC	Cells	FDC	Cells
+	-	-	(few) +

VIRAL LOAD SEMEN
07.01.97
<400 copies/ml

LYMPHOCYTE POPULATION ANALYSIS								
D/M/Y	Lymph.	B.Ly	T-Ly	CD4	CD4 %	CD8	CD8 %	Ratio
09.02.96	1,680	176	1,382	303	19	1,053	66	0.29
22.02.96	2,178	220	1,960	374	17	1,560	71	0.24
26.03.96	1,754	190	1,490	330	19	1,190	68	0.28
01.04.96	2,317	260	2,020	420	18	1,600	69	0.26
08.05.96	1,946	230	1,640	330	17	1,380	71	0.24
30.05.96	2,346	230	1,970	400	17	1,710	73	0.23
22.07.96	1,544	220	1,310	340	22	990	64	0.34
23.08.96	1,640	200	1,390	360	22	1,020	62	0.35
14.10.96	1,830	220	1,537	439	24	1,061	58	0.41
04.12.96	1,447	130	1,270	362	25	897	62	0.40
17.01.97	1,685	185	1,445	438	26	1,078	64	0.41
28.01.97	1,260	126	1,098	290	23	794	63	0.37

Example 4

Sixty HIV-infected subjects, who were asymptomatic and had about 250 to 500 CD4 cells per µl (a normal cell count) and who may have had previous treatment, but not with ddI, were divided into two groups. Group 1 had twenty patients with a dosage of 200 mg ddI twice daily. Group 2 had forty patients with a dosage of 200 mg ddI twice daily plus 500 mg hydroxyurea twice daily. The study duration was twenty-four weeks, with a possible extension to 48 weeks. Figure 5 shows the results for the two populations as variation in plasma viremia over time. The combination of ddI plus hydroxyurea worked better than ddI alone, although a slow downward trend was noticeable, virus was still detectable in the patients blood. In addition, the CD4 cell counts for those treated with the double combination were lower than those receiving only ddI. This raised some cause for concern, as HIV-1 infection typically causes a decline in both the numbers of CD4 cells and their effectiveness. See Fig. 6

Example 5

The study continued with thirty-six patients on the combination of ddI and hydroxyurea. After forty weeks, the curve was either flat or beginning a slow rise. See Fig. 7.

Example 6

After 42 *months* all of the 12 follow-up patients had undetectable (less than 200 copies per milliliter) virus in the blood. Eleven out of twelve had reached undetectable status by about fifteen months. None had rebounded. The shape of the curve is not inconsistent with the hypothesis that a second source of viral particles predominated after about ten months. See Fig. 8.

Example 7

In another study, the combinations of hydroxyurea plus ddI and ddI plus d4T were compared. One group received 40 mg d4T twice daily, 200 mg ddI twice daily, and 500 mg hydroxyurea twice daily. The change in viremia over time shown in Fig. 9, while the change in the CD4 count is shown in Fig. 10. The second group received 40 mg d4T twice daily, 200 mg ddI twice daily, and a placebo. At twelve weeks, 54% of the group

receiving the hydroxyurea combination had fewer than 200 copies of virus per mL, compared to the placebo group. Those with less than 200 copies were then subjected to a more sensitive test, and 19% of the hydroxyurea group had less than twenty copies of virus per mL, while the placebo group had 8% with less than 20 copies of virus per mL. Non-responders (defined as those with more than 200 copies of virus per mL were present in both groups: the hydroxyurea group had 40% and the placebo group had 71%. At twelve weeks, the code of randomization was opened and patients were subsequently treated according to virological response. Thus, a number of patients formerly treated with a placebo were started on hydroxyurea as well, yielding a triple combination of ddl, d4T and hydroxyurea. All combinations showed a drop in virus levels, with the population possibly beginning to rise by the 48th week. When hydroxyurea was added to the combination used by the control group, the level of virus in the blood decreased. In addition the CD4 cell couunts decreased and then increased.

Example 8

Twelve patients were treated with hydroxyurea and didanosine for an average of 122 weeks (range 102-154). Data with respect to CD4 counts, CD8 counts and viral load are shown in Table 7. At the time these patients started the treatment the average baseline plasma viremia measured as viral RNA using a PCR assay with a threshhold level of detection of 200 copies per mL, was 51,795 (SD=66,869) copies/ml (See Fig. 11). At week 40 the average plasma viremia was 1,847 (SD=2,181) copies/ml. At week 122 the average viremia, measured by a supersensitive PCR test having a threshhold level of detection of 50 copies per mL, further decreased to 186 (SD=284) copies/ml. The mean plasma viral RNA

Table 7: Patient characteristics

		Baseline				Last value			
Patient	Weeks	CD4	CD8	ratio	PCR	CD4	CD8	ratio	ss-PCR
1	117	351	n.a.	n.a.	51000	256	330	0.8	265
2	154	397	437	0.9	8553	253	209	1.2	65
3	104	288	794	0.4	173166	392	336	1.2	97
4	113	277	343	0.8	1468	414	504	0.8	<50
5	121	385	n.a.	n.a.	21854	562	783	0.7	<50
6	105	376	811	0.5	13475	640	874	0.7	270
7	110	431	n.a.	n.a.	55521	424	309	1.4	244
8	102	490	1308	0.4	199256	308	986	0.3	915
9	123	493	580	0.9	693	443	424	1.0	<50
10	123	308	736	0.4	66245	314	551	0.6	60
11	141	320	743	0.4	29708	491	685	0.7	119
12	149	399	1033	0.4	602	375	711	0.5	<50
Ave	122	376	754	0.56	51795	406	559	0.83	254*
SD	18	72	294	0.23	66869	118	248	0.31	253*

Weeks: weeks of treatment
PCR: Amplicor PCR, limit of detection = 200 copies/ml
ss-PCR: supersensitive PCR, limit of detection = 50 copies/ml
n.a.: not available; Ave: average; SD: standard deviation
* calculated from the 8 detectable values

decreased by 1.14 logs at week 40 and by 2.2 logs at week 122. Continuous decrease of viremia despite residual viral replication is a novel feature of this two-drug combination. This combination can control HIV over the long term without first achieving maximal virus suppression. No relevant episode of toxicity attributable to the drugs used in this study was described during the average 122 weeks of therapy.

The patient group showed an insignificant increase in the total number of CD4 T-lymphocytes. See Table 7. During the 122 weeks follow up of hydroxyurea and didanosine treatment, the average CD4 count increased by 30 cells/mm^3 from 376$\pm$72 to 406$\pm$118 cells/mm^3. This is consistent with the known characteristics of hydroxyurea, which has cytostatic effects. That is, it tends to suppress cell division. However, the CD4/CD8 ratio significantly increased toward the normal range by 0.27 from 0.56$\pm$0.23 to 0.83$\pm$0.37 ($p=0.039$) (Table 7).

A fundamental question was whether this therapy had beneficial effects on immune parameters and immune function. This group of patients was matched to a group of infected, untreated patients, and the immune systems of the two groups were compared.

After an average 122 weeks of treatment, the 12 patients receiving hydroxyurea and didanosine were matched with a control group of 12 chronically infected patients who declined to accept treatment. Treated and untreated patients were matched based on CD4 count (average$\pm$SD = 406$\pm$118 and 401$\pm$131, respectively, $p = 0.686$), CD8 count (964$\pm$279 and 927$\pm$435, respectively, $p = 0.669$) and plasma viremia (51,795$\pm$66,869 and 51,212$\pm$83,308, respectively, $p = 0.525$). The immune system of each patient was evaluated with respect to the proportion of naive T-lymphocytes and of activated CD8 T-lymphocytes, as well as the vigor of HIV-specific T helper cell response.

It has been shown that the proportion of naive T-lymphocytes, that is, those that have not been exposed to HIV, decreases in patients who have an untreated HIV infection. Consequently, an increase in the proportion of naive T-lymphocytes may be connected with immune system recovery. We found that, after 122 weeks of therapy, the average percentages of naive CD8 and CD4 T-lymphocytes, measured by the expression of CD62L and CD45RA, in patients treated with the HU and ddI combination were similar ($p=0.25$ and $p=0.99$, respectively) to the values of normal uninfected donors

and significantly different (p=0.00053 and p=0.001, respectively) from those of HIV-infected untreated individuals (See Figure 12).

An elevated percentage of activated CD8 T-lymphocytes expressing CD38 and DR indicates a poor prognosis in HIV infection. The percent of activated CD8+CD38+DR+ T-lymphocytes in hydroxyurea plus didanosine treated patients was higher than in normal individuals (p=0.008), but lower than in untreated individuals (p=0.0046), whereas the percentage of CD8+CD28+ cells was lower than in normal individuals (p=0.042) and higher (p=0.0015) than in untreated patients (Figure 13), consistent with a strongly reduced but still residual virus replication. Although an increase of naive T-lymphocytes and decrease of activated CD8 T-lymphocytes have been described after HAART (See Autran, B., Carcelain, G., Li, T.S., Blanc, C., Mathez, D., Tubinana, R., Katalama, C., Debre, P., and Leibowitch, J. (1997). *Science* **277**, 112-6) similar results have been obtained here with the use of only two drugs.

HIV-specific T helper cell responses are associated with control of viremia (Rosenberg, E. S., Billingsley, J. M., Caliendo, A. M., Boswell, S. L., Sax, P. E., Kalams, S. A., and Walker, B. D. (1997) *Science* **278**, 1447-50. HIV-specific CD4 T-lymphocytes proliferative responses are typically absent in persons with progressive infection (Schwartz, D., Sharma, U., Busch, M., Weinhold, K., Matthews, T., Lieberman, J., Birx, D., Farzedagen, H., Margolick, J., Quinn, T., and et al. (1994) *Aids Res Hum Retroviruses* **10**, 1703-11.; Rosenberg, E. S., Billingsley, J. M., Caliendo, A. M., Boswell, S. L., Sax, P. E., Kalams, S. A., and Walker, B. D. (1997) *Science* **278**, 1447-50.) Unfortunately, these responses have not been restored by HAART during the chronic phase of infection (Autran, B., Carcelain, G., Li, T.S., Blanc, C., Mathez, D., Tubinana, R., Katalama, C., Debre, P., and Leibowitch, J. (1997). *Science* **277**, 112-6); Plana, M., Garcia, F., Gallart, T., Miro, J. M., and Gatell, J. M. (1998). *Lancet* **352**, 1194-5.) In contrast, chronically infected patients treated with hydroxyurea and didanosine had a vigorous CD4 T-lymphocytes proliferative response to p24 (Figure 14). It is possible that this response was elicited thanks to the incomplete block of HIV observed in our patients and the consequent constant exposure of HIV antigen to the immune system, whereas current HAARTs might lower viremia to levels so low that the immune system is "off guard" to HIV, "leaving the patient naive to the virus, like an uninfected individual, rather than one prepared to control HIV infection" [Levy, JA. Caution: should we

be treating HIV infection early? *Lancet* 1998;**352**(9132):982-3]. Six out of 12 of these patients had a stimulation index above 9, whereas none of the asymptomatic chronically infected drug-naive patients had a p24 stimulation index above 3.5 (Figure 14). The presence of HIV-specific immune response in chronically infected patients had previously only been described in long-term non-progressors who control HIV in the absence of therapy.

Example 9

In order to test whether normalization of immune functions may lead to control of HIV by an individual's immune system, ten out of twelve patients were allowed to take a six weeks treatment holiday. (See Table 8) Viral load did not increase significantly during the treatment interruption. In contrast, in a HAART treated control group of chronically infected patients viremia rebounded above baseline values during a similarly long interruption. The control group was matched based on baseline viremia (p=0.88), viremia values and CD4 count at treatment interruption (p=0.326 and 0.68, respectively), and number of weeks without therapy (p=0.69). As shown in Table 8, the hydroxyurea-didanosine and the HAART treated groups had similar baseline viremia (average 56,994 and 50,316 copies/ml, respectively), and both treatments suppressed HIV, in most cases to <500 copies/ml. However, after 6 weeks treatment interruption, the average viral load in the hydroxyurea-didanosine treated group remained low (2,680 copies/ml), whereas in the HAART group it rebounded to an average of 136,459 copies/ml, almost 3 fold higher than the original baseline value. The difference between these values was highly significant (p = 0.0001). The average increase from nadir plasma levels in the hydroxyurea and didanosine treated group was only 2,207 copies/ml, still 54,314 copies/ml below the original baseline. HAART treated patients, however, had an average viral load of 136,459 copies/ml, 135,710 copies/ml above the nadir level and 86,143 copies/ml above the original pretreatment baseline.

Table 8: Patients on treatment holidays

Patients	Therapy	Weeks w/o therapy	Baseline VL	Pre-stop CD4	Pre-stop VL	Post-stop VL	VL difference Baseline-Poststop
2	HU, ddI	5	8,553	264	<500	867	-7,686
3	HU, ddI	6	173,166	456	<500	1,542	-171,624
4	HU, ddI	6	1,468	629	<500	<500	-969
5	HU, ddI	6	21,854	630	1,173	2,922	-18,932
6	HU, ddI	6	13,475	556	<500	2,255	-11,220
7	HU, ddI	7	55,521	444	622	5,505	-50,016
8	HU, ddI	3	199,256	251	1,285	2,968	-196,288
9	HU, ddI	6	693	346	<500	<500	-194
10	HU, ddI	7	66,245	380	597	7,632	-58,613
11	HU, ddI	6	29,708	430	<500	2,106	-27,602
	Average	5.8	56,994	439	667*	2,680	-54,314
	SD	1.1	71,721	456	301*	2,291	71,279
21	AZT,RTV,NVP	9	70,000	346	<500	78,600	8,600
22	AZT,3TC,IDV	4	7,358	360	<500	24,000	16,642
23	AZT,ddC,SQV	6	7,800	183	<500	42,000	34,200
24	AZT,ddC,RTV	6	8,700	521	<500	222,000	213,300
25	d4T,ddC,SQV	5	37,900	440	<500	107,920	70,020
26	d4T,ddI,IDV	3	50,000	520	<500	55,000	5,000
27	d4T,3TC,RTV,SQV	9	35,000	267	<500	288,000	253,000
28	ddI,3TC,RTV,SQV	4	160,000	553	<500	84,070	-75,930
29	d4T,3TC,IDV	4	10,400	375	<500	25,000	14,600
30	AZT,3TC,(IDV)	9	116,000	616	3,000	438,000	322,000
	Average	5.9	50,316	418	749*	136,459	86,143
	SD	2.3	51,747	136	791*	136,928	129,705

Weeks w/o therapy: weeks beetween interruption of the treatment (pre-stop) and reinitiation of the treatment (post-stop)

* for calculations <500 was interpreted as 499

Example 10

Three rhesus macaques infected with SIV/Mac251 (baseline viremia 198,893, 548,513 and 1,254,156 copies/ml) were treated 28 days post-infection with hydroxyurea, ddl, and a substitute for a protease inhibitor, PMPA. Treatment was withdrawn twice, and the course of the infection was followed up by plasma viremia analysis. The results obtained were compared to the results obtained with patient BM. Protease inhibitors cannot be used on primates, so PMPA, a reverse transcriptase inhibitor which is known to affect primates in the manner that protease inhibitors affect human beings, was used as a substitute. The results are shown in Figs. 15-19. Fig. 15 contains information from patient BM adapted to the format of the present set of figures for ease of reference. The primary difference between this figure and Fig. 4 is that day zero is taken to be the suspected date of infection, rather than the first day of treatment. Figs. 16, 17, and18 report the results for the monkeys individually, and Fig. 19 reports the consolidated results for all three monkeys.

The first animal experienced viral rebound during the first treatment discontinuation only. The second animal experienced rebound during the second treatment discontinuation only. The third animal experienced rebound in response to both. However, rebound in each animal was controllable, either by drug treatment or by the individual's immune system in the absence of drug treatment. After the second discontinuation, all three animals eventually developed an undetectable level of viremia in the absence of therapy. Viremia has remained undetectable in these animals.

Example 11

Three human patients were tested using intermittent therapy as described above. The patients had viremia levels of 16,130, 21,845 and 719,000 copies/mL, as well as CD4+ counts of 264, 508 and 880, respectively. Two of the patients were put on drug combinations including Hydroxyurea and ddl plus a protease Indinavir as described in Example 2 above. The third began with the same combination, but developed peripheral neuropathy, and adverse reaction which might be attributable to treatment with ddl, during a break in treatment. This patient was then treated with a variety of antiviral drugs. The patients had three weeks of triple combination therapy, followed by 1 week treatment interruption. Then,

treatment was begun again for three months, followed by a second interruption. The second interruption was followed by another three months of therapy and a third interruption. Patient A has not yet reached to third interruption, so this data point is not yet available. Unlike the animals, the interruption period was cut short, and therapy restarted as soon as rebound was established (when the plasma viremia level reached about 5,000 copies/mL). The time without rebound was measured and is shown in Fig. 20. It was found that the time to rebound increased logarithmically with each hiatus in treatment.

Example 12

Introduction

To study the outcome of 8 weeks structured treatment interruptions (STI) in chronically infected individuals, a cohort of patients (PANDAs) treated with hydroxyurea and didanosine therapy was matched to a cohort of HAART treated patients. Matching criteria were: length of treatment, CD4 and CD8 counts. Patients were scheduled to interrupt treatment for 8 weeks or earlier, if failure, defined as viremia rebound >10,000 copies/ml or CD4 count decrease below 200 cells/mm3, occurred. Structured treatment interruptions (STI) (Lori F, Maserati R, Foli A, Seminari E, Timpone J, Lisziewicz J. Structured Treatment Interruptions to Control HIV. *The Lancet* 1999; **in press.**) are attractive alternatives to continuous treatments, since they could reduce toxicity and improve the patients' quality of life.

Concerns exist, however, that STI might expose the virus to sub-optimal drug concentrations (thus increasing the possibility of the onset of resistant mutants and virus escape), and might expose the patients to undesirable boosts of viral replication. In fact, interruption of HAART is usually followed by a loss of CD4 cells and a rapid viral rebound (de Jong MD, de Boer RJ, de Wolf F, et al. Overshoot of HIV-1 viraemia after early discontinuation of antiretroviral treatment. *AIDS* 1997;**11**(11):F79-84. Staszewski S, Miller V, Sabin C, Berger A, Hill AM, Phillips AN. Rebound of HIV-1 viral load after suppression to very low levels. *AIDS* 1998;**12**(17):2360. Jubault V, Burgard M, Le Corfec E, Costagliola D, Rouzioux C, Viard JP. High rebound of plasma and cellular HIV load after discontinuation of triple combination therapy. *AIDS* 1998;**12**(17):2358-9. Neumann AU, Tubiana R, Calvez V, et al. HIV-1 rebound during interruption

of highly active antiretroviral therapy has no deleterious effect on reinitiated treatment. Comet Study Group. *AIDS* 1999;**13**(6):677-83. Harrigan PR, Whaley M, Montaner JS. Rate of HIV-1 RNA rebound upon stopping antiretroviral therapy. *AIDS* 1999;**13**(8):F59-62.), also in those instances where the HIV latent reservoir has been undetectable. (Chun TW, Fauci AS. Latent reservoirs of HIV: obstacles to the eradication of virus. *Proc Natl Acad Sci U S A* 1999;**96**(20):10958-61.) Therefore, we investigated whether viral load rebound and CD4 decrease could be contained during STI.

The PANDA cohort consists of chronically infected patients treated with hydroxyurea and didanosine who suppressed HIV replication over two years in the absence of viral breakthrough. The profile of viral load reduction was unusual for a two-drug combination, since a continuous gradual decrease of viremia persisted despite residual viral replication. Similar to patients on HAART, PANDAs normalized the percentages of naïve CD4 and CD8 T lymphocytes. However, unlike those of patients treated with other therapies, vigorous HIV-specific T-helper responses were detected in the PANDAs. Given the unique profile of this cohort, we decided to test the feasibility of STI in the PANDA cohort.

Methods

Patients: The patients described in this study as PANDAs have been long-term treated with the combination of hydroxyurea and didanosine. This cohort was matched with a cohort of asymptomatic patients treated with HAART, based on the length of treatment, CD4 and CD8 count, as described in Table 1.

Statistical methods: Groups were matched by using the Mann-Whitney nonparametric U Test.

Surrogate markers: Plasma viremia was measured with supersensitive PCR according to Bagnarelli et al. (Bagnarelli P, Valenza A, Menzo S, et al. Dynamics of molecular parameters of human immunodeficiency virus type 1 activity in vivo. *J Virol* 1994;**68**(4):2495-502.) (detection limit = 50 copies/ml). CD4 and CD8 counts were performed according to standard protocols.

Results

After an average 151+17 weeks of hydroxyurea and didanosine therapy, ten PANDAs accepted to interrupt treatment for 8 weeks. Before interruption, average viremia was 746+710 copies/ml. Six weeks after STI, viremia increased to 26,000+63,749 copies/ml, then unexpectedly decreased to an average of 2,883+3,657 copies/ml at week 8 (Fig. 21). After therapy restart, the viral load returned to values similar to those before treatment interruption (Fig. 21). These results were in contrast to those described after interruption of HAART, therefore we decided to repeat the STI in a matched controlled study.

The PANDA group was matched with a group treated with HAART (Table 9). The matching criteria were length of previous therapy (>100 weeks in both groups), CD4 count and CD8 count. Viremia, however, was different between the two groups. Seven of eight HAART patients had viremia below 50 copies/ml, whereas only one of nine PANDAs had viremia below 50 copies/ml. PANDAs and HAART patients signed informed consent to interrupt treatment for a maximum of 8 weeks. However, treatment would be restarted earlier if viral load rebounded above 10,000 copies/ml (in 2 consecutive tests), or CD4 count decreased below 200 cells/mm3. By week 2, one HAART patient had to restart therapy because of viremia rebound (Fig. 22). By week 6, other four HAART patients had to restart therapy: three of them because of viral rebound and one of them because of CD4 decrease. In contrast, none of the nine PANDA patients had to restart therapy during the 8 weeks of STI.

Table 9. Matched cohorts

	Patient	Treatment	Weeks on therapy before Stopping	Viral Load (copies/ml)	CD4 Cell Count (cells/µl)	CD8 Cell Count (cells/µl)
HAART	1	d4T+3TC+SQV+RTV	86	91	789	646
	2	d4T+3TC+SQV+RTV	139	< 50	1079	1140
	3	d4T+3TC+SQV+RTV	113	388	428	1147
	4	d4T+3TC+SQV+RTV	99	< 50	341	251
	5	d4T+3TC+SQV+RTV	121	< 50	290	743
	6	d4T+3TC+IDV	118	< 50	511	800
	7	d4T+3TC+IDV	108	< 50	616	1000
	8	AZT+3TC+SQV+RTV	67	< 50	428	876
	Average		**106**	**97**	**560**	**825**
	SD		**23**	**119**	**263**	**294**
PANDAs	9	HU+ddl	211	1500	235	245
	10	HU+ddl	162	242	540	1297
	11	HU+ddl	177	144	572	952
	12	HU+ddl	179	1071	532	1184
	13	HU+ddl	163	445	662	1037
	14	HU+ddl	169	395	393	393
	15	HU+ddl	198	< 50	600	560
	16	HU+ddl	189	549	461	762
	17	HU+ddl	207	548	462	816
	Average		**184**	**549**	**495**	**805**
	SD		**18**	**464**	**127**	**355**

d4T: stavudine; 3TC: lamivudine; SQV: saquinavir; RTV: ritonavir; IDV: indinavir, AZT: zidovudine; HU: hydroxyurea; ddl: didanosine. SD: standard deviation.
Calculations are performed assuming that <50 = 49.

Next we analyzed the average changes of viremia, CD4 count, CD8 count, and CD4/CD8 ratio throughout 8 weeks of STI in the PANDA cohort. The same parameters were analyzed throughout only 6 weeks of STI in the HAART cohort, since most of these patients had to restart therapy after 6 weeks. Viremia changes in the PANDA group showed a pattern similar to that observed during the 1st interruption. After an initial increase of viremia (from average 549+464 copies/ml at baseline to average 4,185+5,299 copies/ml at week 2), viral rebound was partially controlled, and viremia progressively decreased during the next 6 weeks (Fig. 23). At week 8 of STI, average viremia was 1,596+1184 copies/ml (change above baseline = +0. 46 log). In contrast, viremia in HAART patients rebounded to +2.24 log above baseline after 6 weeks of STI (from average 97+119 copies/ml at baseline to average 16,863+31,915 copies/ml at week 6).

Consistent with the viremia profile, CD4 count only slightly decreased in the PANDA cohort during STI (from 495+127 cells/mm3 at baseline to 432+116 cells/mm3 at week 8) (Fig. 24). However, CD4 count considerably decreased in the HAART patients during STI (from 560+263 cells/mm3 at baseline to 409+293 cells/mm3 at week 6). CD8 count was stable in the PANDA cohort (805+335 cells/mm3 at baseline and 808+343 cells/mm3 at week 8 of STI) whereas it increased in the HAART cohort from 851+308 cells/mm3 at baseline to 1106+447 cells/mm3 at week 6 of STI (Fig. 25). CD4/CD8 ratio significantly (p=0.01) decreased in HAART patients after treatment interruption (from 0.66+ 0.38 at baseline to 0.37+0.21 at week 6 of STI). In contrast, CD4/CD8 ratio did not significantly changed in the PANDAs during the 8 weeks of STI (0.62+ 0.24 cells/mm3 at baseline and 0.54+0.3 cells/mm3 at week 8)(Fig. 26).

In both groups, viremia decreased to the pretreatment values after treatment was restarted. (Fig. 21) Twelve weeks after reinitiating therapy the average viremia was 355+321 copies/ml in the PANDAs and 54+13 copies/ml in the HAART cohort. CD4 count also returned to baseline values in the HAART patients (538+448 cells/mm3) and it was maintained at 477+133 cells/mm3 in the PANDAs. CD8 count remained unchanged (801+312 cells/mm3) in the PANDAs, while it decreased to 1048+653 cells/mm3 in the HAART patients.

Conclusions

In this prospective study we confirmed that interruption of long and successful HAART in chronically infected patients results in a rapid rebound of viremia, decline of CD4 count and CD4/CD8 ratio in most of the patients. We also confirmed that re-initiation of HAART promptly reduces viral load down to values similar to those before therapy discontinuation. Unlike most HAART patients, chronically infected patients long-term treated with hydroxyurea and didanosine (PANDAs) contained the viremia rebound during two consecutive STI. In both STI, an initial rebound was followed by a partial control of viremia. Consistent with the viremia profile, only marginal changes of CD4 count, CD8 count and CD4/CD8 ratios were observed in the PANDAs.

Although partial control of viremia was observed in both the arms of the study, the results suggest that the rate of success might be dependent on the antiretroviral regimen used prior to the STI. Previous observations might explain these results: 1) hydroxyurea has cytostatic and immune-modulatory properties. It has been proposed that the use of a cytostatic drug might prevent viral rebound by reducing the availability of activated targets ("preys") for HIV ("predator") infection; 2) didanosine and hydroxyurea are most potent anti-HIV drugs in infected macrophages, and dendritic cells. These cells are HIV reservoirs that might be responsible for re-ignition of viral replication; 3) Long-term exposure to low levels of HIV in the hydroxyurea and didanosine treated patients might have been critical to induce HIV-specific T-cell mediated immunity previously observed in the PANDAs. In HAART patients, characterized by undetectable viremia, these responses usually disappear, leaving these patients naïve to the virus, thus unable to control HIV after treatment interruption.

In sum, none of the PANDAs failed during 8 weeks STI. The initial viremia rebound was spontaneously contained in all PANDAs (average +0.46 log from the baseline at week 8 STI). CD4 and CD8 counts remained stable during STI in these patients. In contrast, five of eight HAART patients failed to control HIV by week 6 and had to restart therapy. In HAART treated patients viremia rebounded by week 6 to an average +2.25 log from the baseline, CD4 count decreased and CD8 count increased during STI. This experiment shows that control of HIV during STI is achievable in chronically infected patients and the rate of success might depend on the antiretroviral regimen used for treatment.

Example 13

The infection of rhesus macaques by Simian Immunodeficiency Virus (SIVmac251) was chosen as an animal model because of the similarities of SIV in macaques to HIV infection in humans. Mucosal inoculation of macaques with SIVmac251 reproducibly resulted in an infection characterized by peak plasma viremia within 2-3 weeks post infection, followed by a plateau which can persist for several months. Eventually, most animals progress toward an acquired immune deficiency syndrome, although, occasionally, a low percentage of infected animals manage to spontaneously control virus replication and exhibit very low levels of plasma viremia, similar to human long-term non progressors. Studies of antiretroviral therapy have been limited until recently when PMPA was shown to effectively inhibit SIV replication in this non-human primate model. Protease inhibitors do not work in an SIV infected monkey model. Therefore, we have used the combination of PMPA, ddI and HU as HAART, because our preliminary experiments demonstrated that this combination can rapidly and effectively decrease viral load in SIV-infected animals.

A total of 29 rhesus macaques were infected via mucosal (intra-rectal) inoculation with SIVmac251 (5.12x103 TCID 50 in 3ml). The combination of PMPA (20 mg/Kg once daily subcutaneously), ddI (10 mg/Kg once daily intravenously), and HU (15 mg/Kg once daily intravenously) was selected because preliminary experiments had shown that this combination can effectively suppress SIV viral load for long periods of time, similar to HAART in HIV infected humans. A group of five SIV infected and untreated animals served as controls. A group of six SIV infected animals received continuous antiretroviral therapy initiated 44 days post infection. The other 3 groups were treated intermittently for a total of 24 weeks. The groups treated intermittently were on the same schedule, 3 weeks on followed by 3 weeks off. In sum, Group # 1 was untreated, Group #2 was treated with intermittent therapy, (HU + ddI + PMPA); Group #3 was treated with intermittent therapy that did not include hydroxyurea, (ddI + PMPA); Group #4 was treated with intermittent therapy for two drugs, ddI and PMPA, and continuous therapy for a third, hydroxyurea (ddI + PMPA , intermittent, HU continuous); Group #5 was treated continuous therapy (HU+ ddI + PMPA, continuous treatment).

STI is superior to HAART for Treatment of viral diseases

Fig. 27 shows the viral load for all monkeys from shortly before therapy was begun until about one month after therapy ended. Fig. 28 shows the viral load for the same monkeys at baseline, during therapy, and 41 days after cessation of therapy. Because their results are similar, all the monkeys treated with STI are shown in Fig. 28 as a single group. The virology of this experiment demonstrates that both treatment schedules, continuous HAART and STI, decreased the viral load efficiently after introduction of therapy. Compared to the untreated control, the viral load in all cases was either undetectable or at a very low level during the treatments. The differences among the three STI therapies with respect to the maintenance of viral load were also insignificant during the treatment.

This picture changed dramatically after permanent treatment interruption. The viral load of the animals rebounded in the group treated continously with HAART (cont ddI+PMPA+HU) and one animal died one month after therapy interruption. No animals died in the untreated control group. This was not surprising, because it is known that after interruption of HAART, viral load rebounds to the pretreatment values or higher, even if it starts from a very low undetectable level. In contrast, the monkeys treated with STI controlled SIV replication at least 2 months after permanent interruption of therapy. In the group of STI(ddI+PMPA+HU) the results were dramatic: 6 of the 6 animals controlled SIV. In each of the two other groups of STI(ddI+PMPA) and STI(ddI+PMPA+cont HU) one animal was a non-responder (never respond to therapy, a finding not uncommon in the treatment of these animals, irrespective of the kind of treatment administered), one animal's viral load rebounded, and 4 animals controlled SIV. These results demonstrate that (1) continous HAART cannot be interrupted because viral load rebounds rapidly and, more importantly, after therapy interruption, patients have a higher risk of dying than if they had remained untreated; (2) Intermittent therapy (STI) can control viral replication after therapy discontinuation; (3) Hydroxyurea is a useful but not essential component of HAART used for STI.

CD4 counts

A patient's CD4 counts typically decrease if HIV infection is untreated. One concern with HU-containing therapies was that although these therapies decrease the viral load, significant increases in the

patient's CD4+ T cell count are generally not observed. Here we studied CD4 counts in our monkey model.

Fig. 29 shows the number of CD4+ lymphocytes for 29 monkeys at initiation of therapy, during therapy, and 41 days after cessation of therapy.

Our results confirm that the course of infection in the monkey model is similar to that of HIV in that CD4 cell counts consistently decrease over time during SIV infection in the absence of treatment. Both continuous HAART and STI can increase the CD4 count. At the end of therapy, no differences in CD4 cell counts were observed between the continuous HAART and STI groups. This is consistent with the viral load analysis and provides further evidence that that STI is as effective for treatment as continuous HAART.

After cessation of continuous HAART, the CD4 counts began to decrease rapidly. At 41 days after treatment interruption, the CD4 counts of animals treated with continuous HAART were no different from the CD4 counts of untreated animals. The CD4 count and the viral load data provide evidence that patients treated with continuous HAART lose the benefits gained during therapy. Since we had one death in the HAART group, it is also possible that continuous HAART treatment is worse than no treatment if the therapy has to be permanently interrupted, as might be the case when a drug has toxic side effects. It is notable that, 41 days after permanent discontinuation of STI, the CD4 counts had not decreased significantly. This data provides additional evidence that a virus can be controlled after permanent discontinuation of STI.

<u>STI is less toxic than continuous HAART</u>

Bone marrow toxicity, known to be associated with the use of HU, was closely monitored. Two animals in the continuous HAART group experienced a slight decrease in the hemoglobin levels (from 11.9 g/dL to 9.2 g/dL in animal #19196, and from 13.1 g/dL to 11.1 g/dL in animal #19152). This mild toxicity was attributed to the use of HU, however, it did not warrant any modification of HU dosage. Bone marrow toxicity was more severe in the untreated controls. In three animals (#716, #19763, and # 19766) hemoglobin levels decreased from 13.1 g/dL to 10.5 g/dL, from 12.8 g/dL to 9.2 g/dL, and from 12.8 g/dL to 8.0 g/dL, respectively. This decrease probably reflected the SIV-mediated bone marrow toxicity. Unexpectedly, five months after treatment initiation, technicians noted a

visible decline in the health of some animals treated with continuous HAART therapy. Blood tests revealed that five of six animals had increased glucose levels in the plasma (range 231 to 448 mg/ml) and one of them (animal #19197) also had increased transaminase levels (AST=448 IU/L, and ALT=382 IU/L). In contrast, the infected untreated animals had normal values, indicating that the toxicity was due to the use of the antiretroviral drugs.

Administration of all drugs was interrupted for the group. Despite the treatment interruption, the clinical conditions deteriorated. Two animals (#19197 and#19720) were reported as depressed in their cages, hunched, and anorexic. They also had weight loss of 0.5 to 1.0 Kg from the previous week, mainly due to dehydration. Both animals received fluid therapy and nutritional support. Two other animals (#19196 and #19512) had abdominal distension, visible wasting and increased output of clear urine. Two weeks after treatment interruption, glucose levels in five of six animals (#19720, #19197, #19152, #19196, and #19729) were severely elevated (758-1455 mg/ml) (Table 10). Four of these animals had increased aspartate aminotransferase (AST) levels (245-10450 IU/L), and three also had increased alanine aminotransferase (ALT) levels (246-780 IU/L). Two of the animals showed increased amylase levels (746 and 2134 IU/L). Alkaline phosphatases were very high in one animal (3090 IU/L) and moderately elevated in another one (793 IU/L). Lipases were significantly elevated in one animal (623 IU/L), and slightly elevated in two other animals (Table 10). The sixth animal (#710) had glucose levels slightly above normal (108 mg/ml), and amylases were also elevated (746 IU/L). Insulin treatment (recombinant-human DNA derived, at 2 IU/Kg) was promptly started in all animals, except in animal # 710. One week later the conditions of the two animals (#19720 and#19197) that were most ill stabilized. The animals became eager to eat and much more active. Glucose and transaminase levels significantly decreased. All five animals exhibited severe muscle wasting, weakness polyuria and polydypsia. Insulin treatment, nutritional and supportive care were continued. Note, that these are common toxicities in HAART treated patients.

Table 10. Laboratory values of 6 rhesus macaques continuously treated with PMPA, ddI and HU

2 weeks after treatment interruption							
Animal #	19720	19197	19152	19196	19729	710	(Normal)
GLU	1455	977	950	758	908	108	33-95
ALT	780	473	129	246	188	41	18-204
AST	10450	245	124	596	373	50	23-175
ALK P	793	3090	304	231	239	526	65-641
LDH	1705	498	575	749	1326	747	578-4603
TRIG	44	60	218	164	79	83	23-194
AMYL	137	139	2134	495	357	746	178-551
LIP	222	363	623	167	181	130	30-190

In striking contrast to what happened in these animals, no toxic side effects were observed in any of the animals that received STI treatment.

WE CLAIM:

1. A method of inducing autovaccination in an individual by an autologous human immunodeficiency virus comprising the steps of: administering to the individual a drug therapy effective to restrict viral replication in the presence of detectable levels of the virus for at least about thirty-six weeks, whereby the individual is continuously exposed to sufficient antigen to induce HIV-specific immune response.
2. The method of Claim 1, wherein the drug therapy comprises an effective amount of hydroxyurea and a reverse transcriptase inhibitor.
3. The method of Claim 2, wherein the reverse transcriptase inhibitor is selected from ddI, d4T, 3TC, AZT, delavirdine, abacavir, adefovir, nevirapine, efavirenz, lubocavir , PMPA PMEA, and mixtures thereof.
4. The method of Claim 1, further comprising the step of administering during the course of treatment at least one agent for activating quiescent cells harboring the virus.
5. The method of Claim 1, wherein the antigen is HIV RNA particles in the plasma, and the amount of particles is about 500 ± 1 log to 50 ± 1 log.
6. A method of inducing autovaccination by autologous human immunodeficiency virus, the steps comprising
   administering a drug therapy combination which can control a human immunodeficiency virus population to levels of less than 200 copies per mL within a six week period,
   interrupting administration of the drug combination,
   monitoring the individual for viral rebound,
   restarting the drug therapy when the viral load in the plasma increases to about 5,000 copies per mL.
7. The method of Claim 6, wherein the drug combination comprises a highly active retroviral drug therapy.
8. The method of Claim 7, wherein the drug combination is hydroxyurea, one or more reverse transcriptase inhibitors, and one or more protease inhibitors.
9. The method of Claim 8, wherein the reverse transcriptase inhibitor is selected from ddI, d4T, 3TC, AZT, delavirdine, abacavir, adefovir, nevirapine, efavirenz, lubocavir , PMPA PMEA, and mixtures thereof.

10. The method of Claim 8, wherein the protease inhibitor is selected from Indinavir, saquinavir, ritonavir, Nelfinavir, GW141, and mixtures thereof.

11. The method of Claim 8, further comprising the step of administering during the course of treatment at least one agent for activating quiescent cells harboring the virus.

Fig. 1

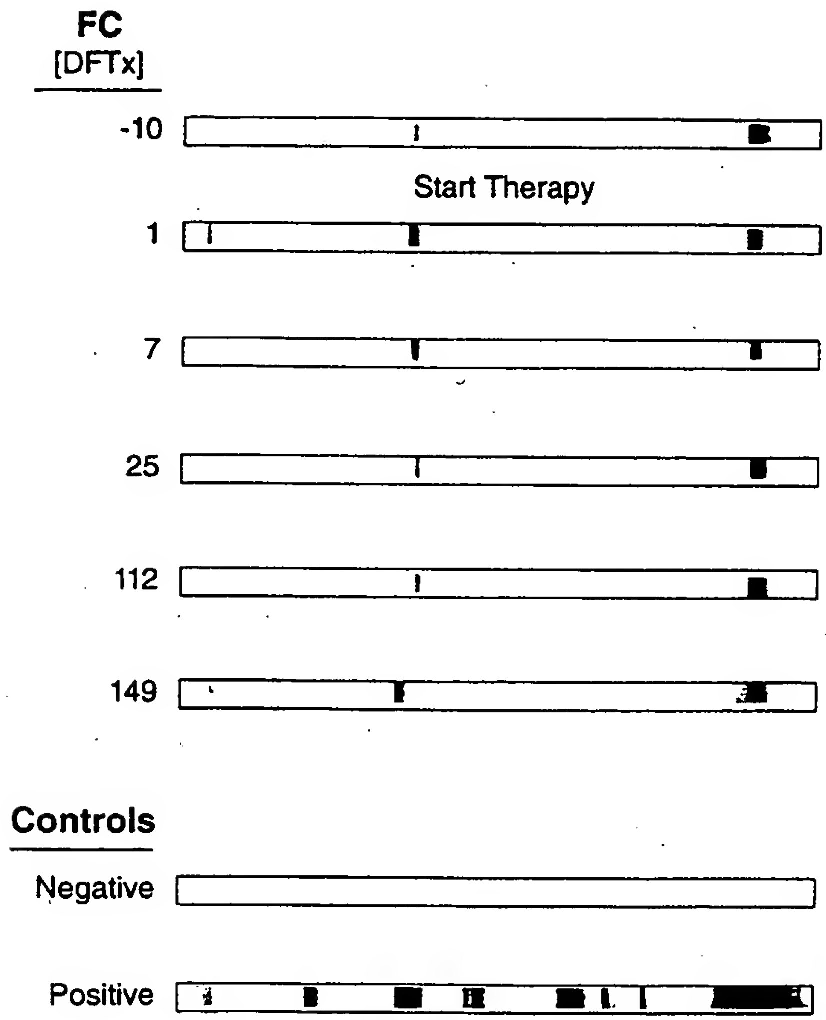

FC
[DFTx]
-10
Start Therapy
1
7
25
112
149
Controls
Negative
Positive

Fig. 2

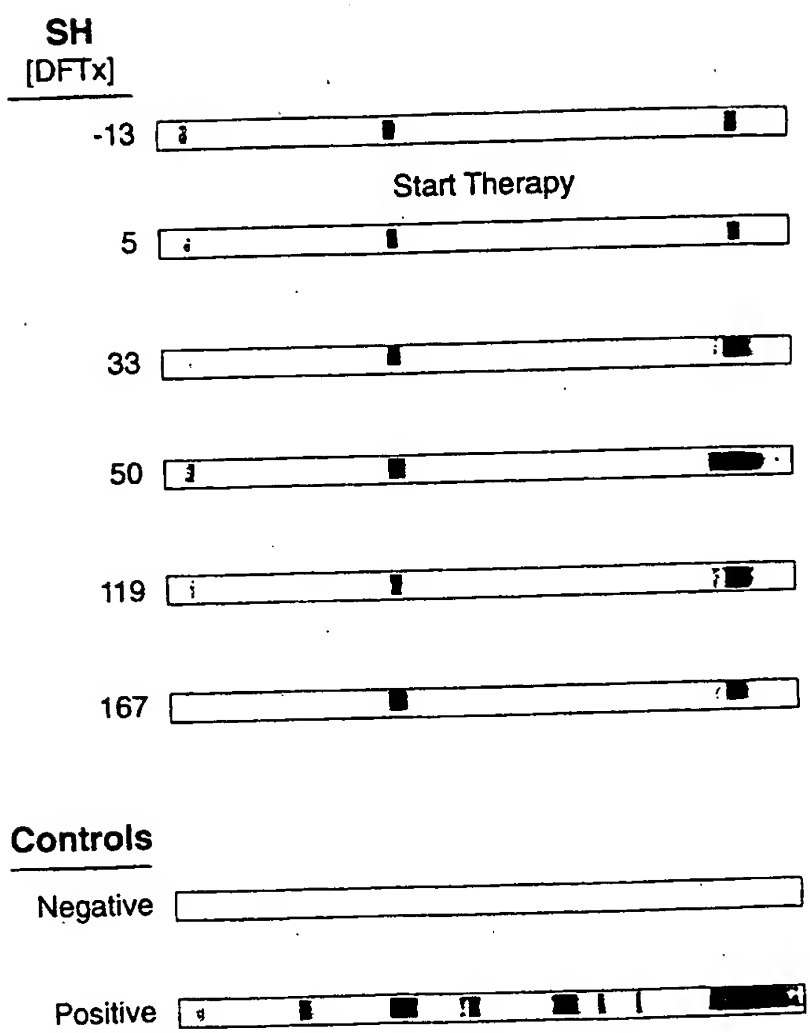

SH
[DFTx]
-13
Start Therapy
5
33
50
119
167
Controls
Negative
Positive

Fig. 3

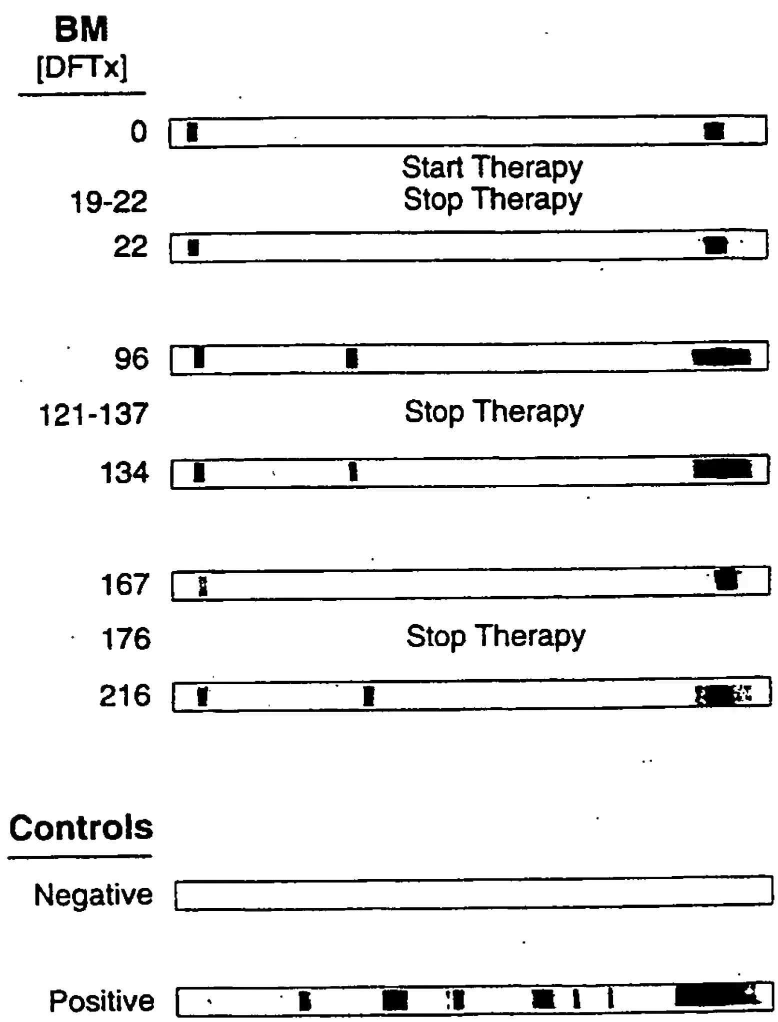
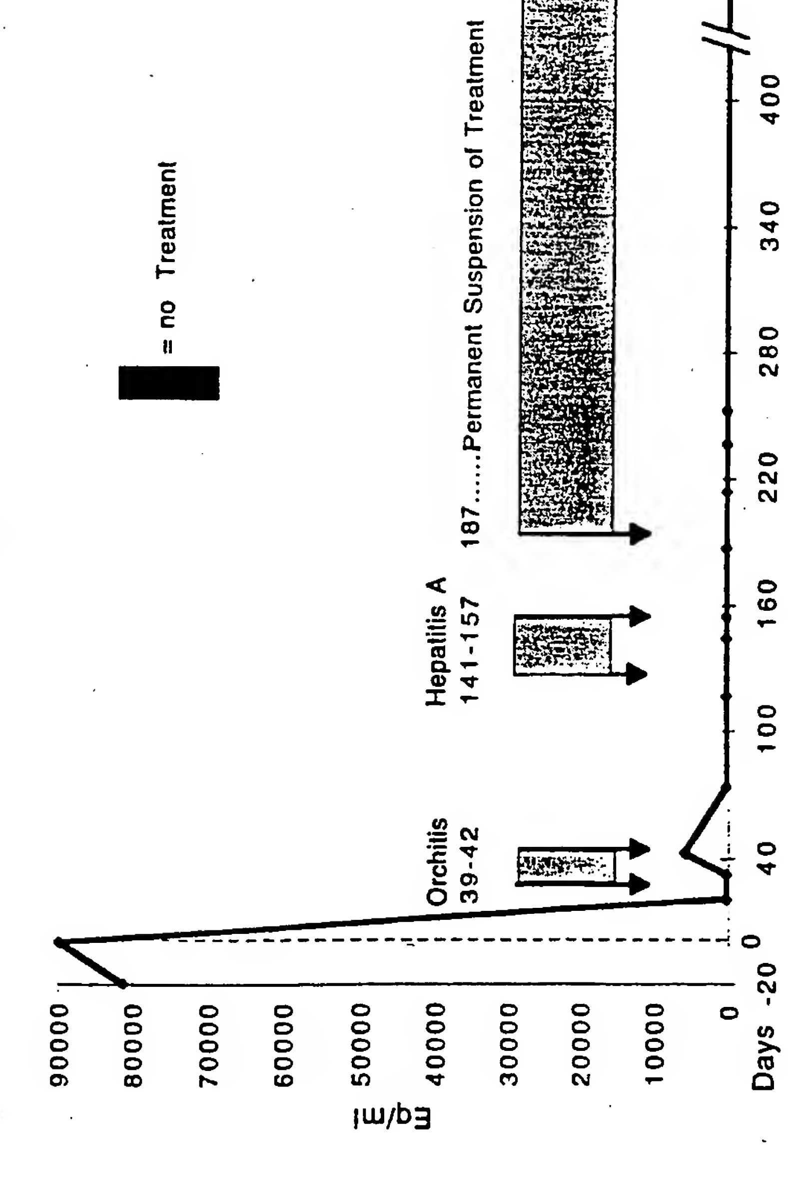

BM
[DFTx]
0
Start Therapy
19-22
Stop Therapy
22
96
121-137
Stop Therapy
134
167
176
Stop Therapy
216
Controls
Negative
Positive

Fig. 4



	Day 112	Day 265	Day 582
Semen viremia		undetectable	
Lymphnode HIV DNA		undetectable	
Lymphnode HIV RNA	2 cells/44 million	3 cells/44 million	
Replication competent HIV			<1 cell/10 million

Fig. 5

Combination of HU + ddI

Study n. 1 24 weeks

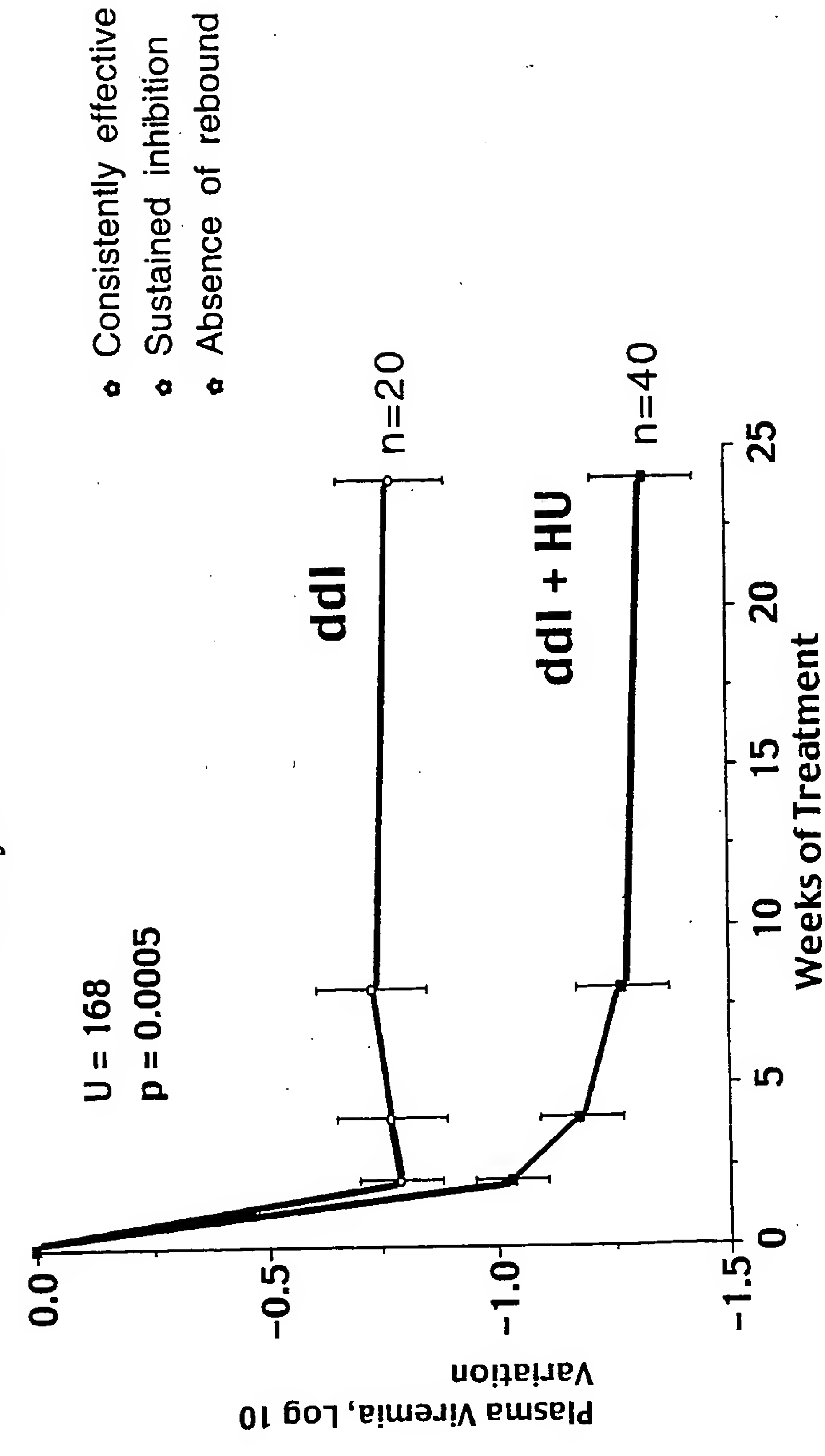


Fig. 6

CD4 Cell Count does not Increase Significantly after HU+ddI Treatment

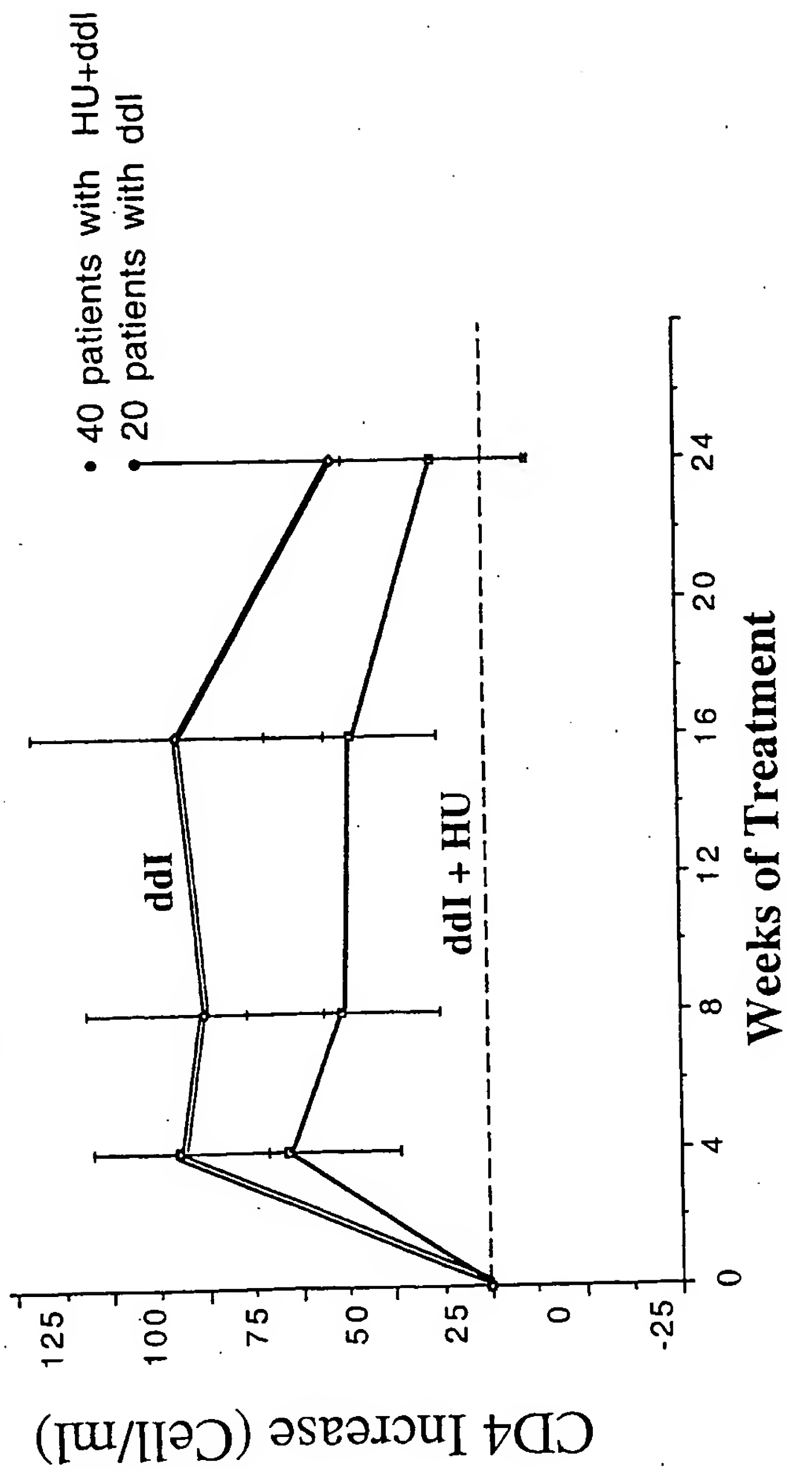


Fig. 7

Combination of HU + ddI
Study n. 1 40 weeks

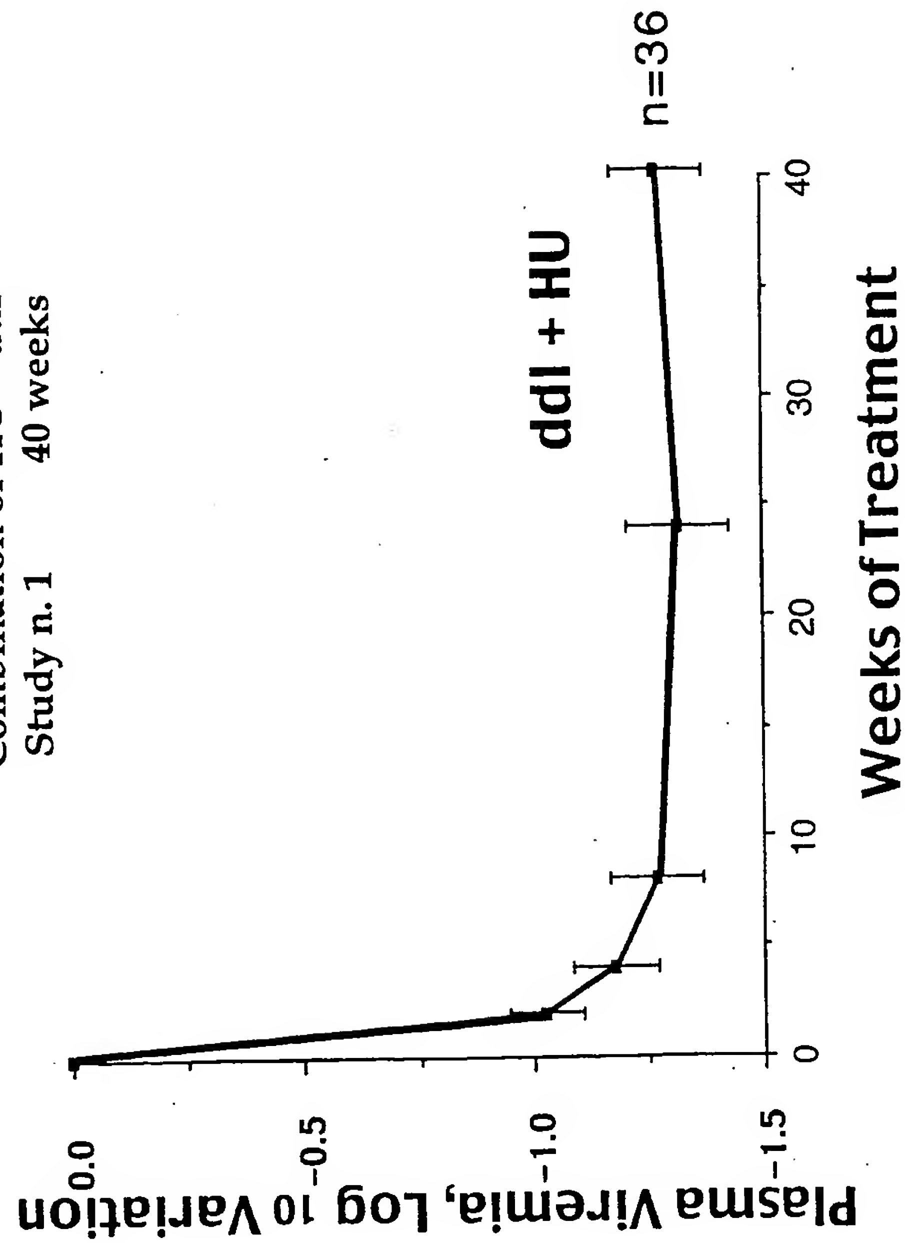


Fig. 8

**Plasma Viremia after Long-Term HU+ddI Therapy
12 Patients Follow-up (Average 28 months)**

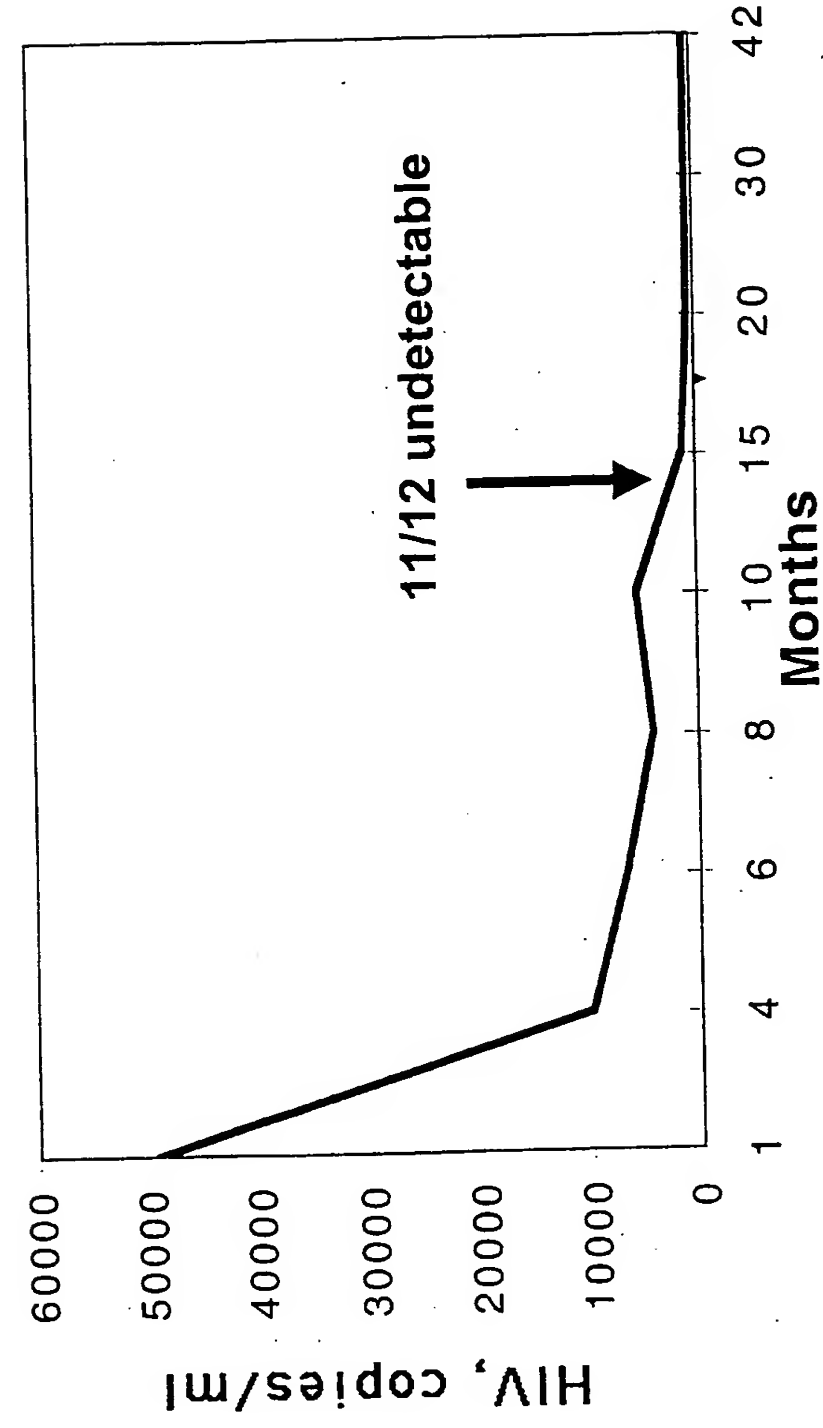


Fig. 9

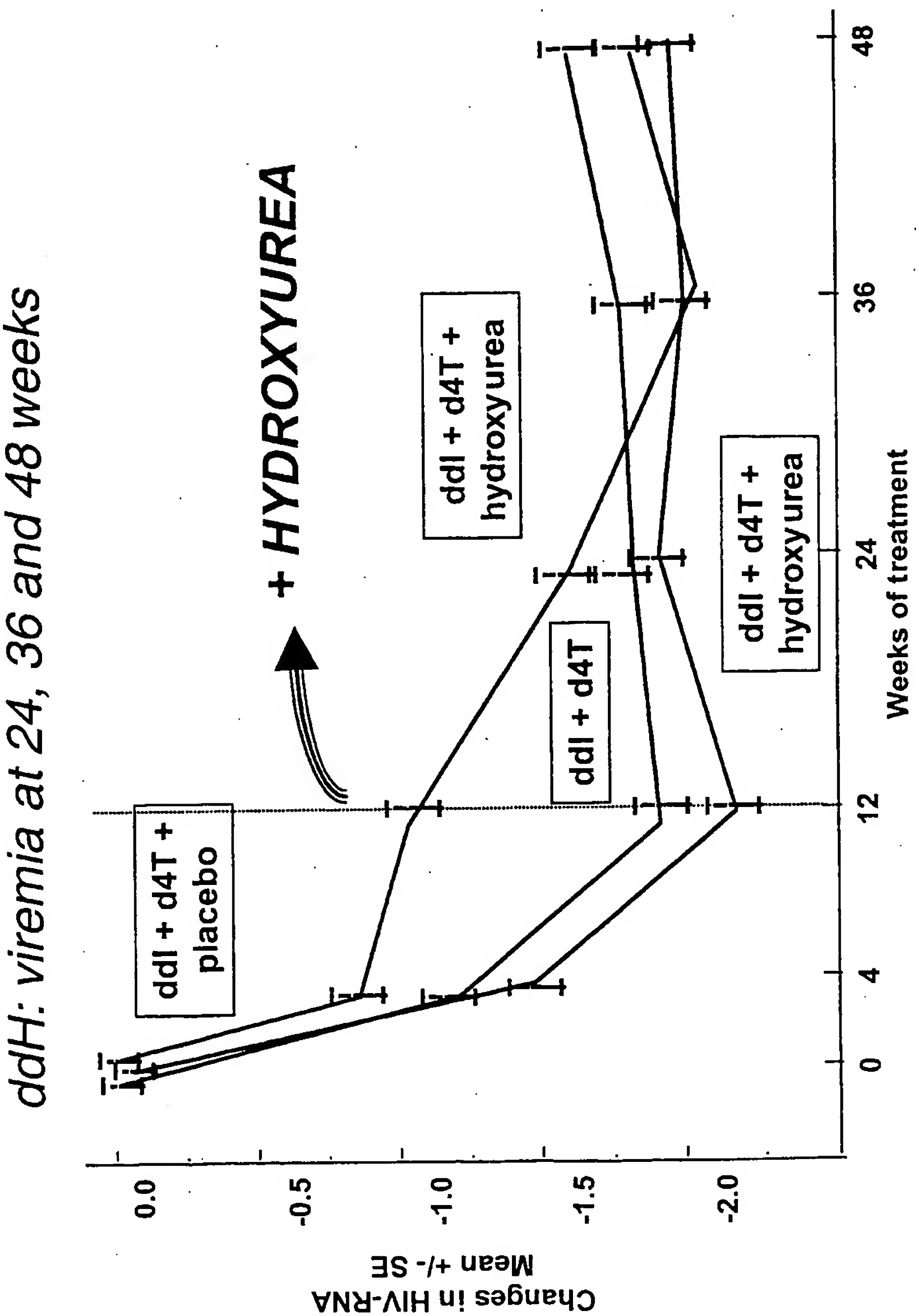

ddH: viremia at 24, 36 and 48 weeks
+ HYDROXYUREA
ddI + d4T + placebo
ddI + d4T
ddI + d4T + hydroxyurea
ddI + d4T + hydroxyurea
Changes in HIV-RNA Mean +/- SE
0.0
-0.5
-1.0
-1.5
-2.0
0
4
12
24
36
48
Weeks of treatment

Fig. 10

ddH: CD4 at weeks 24, 36 and 48

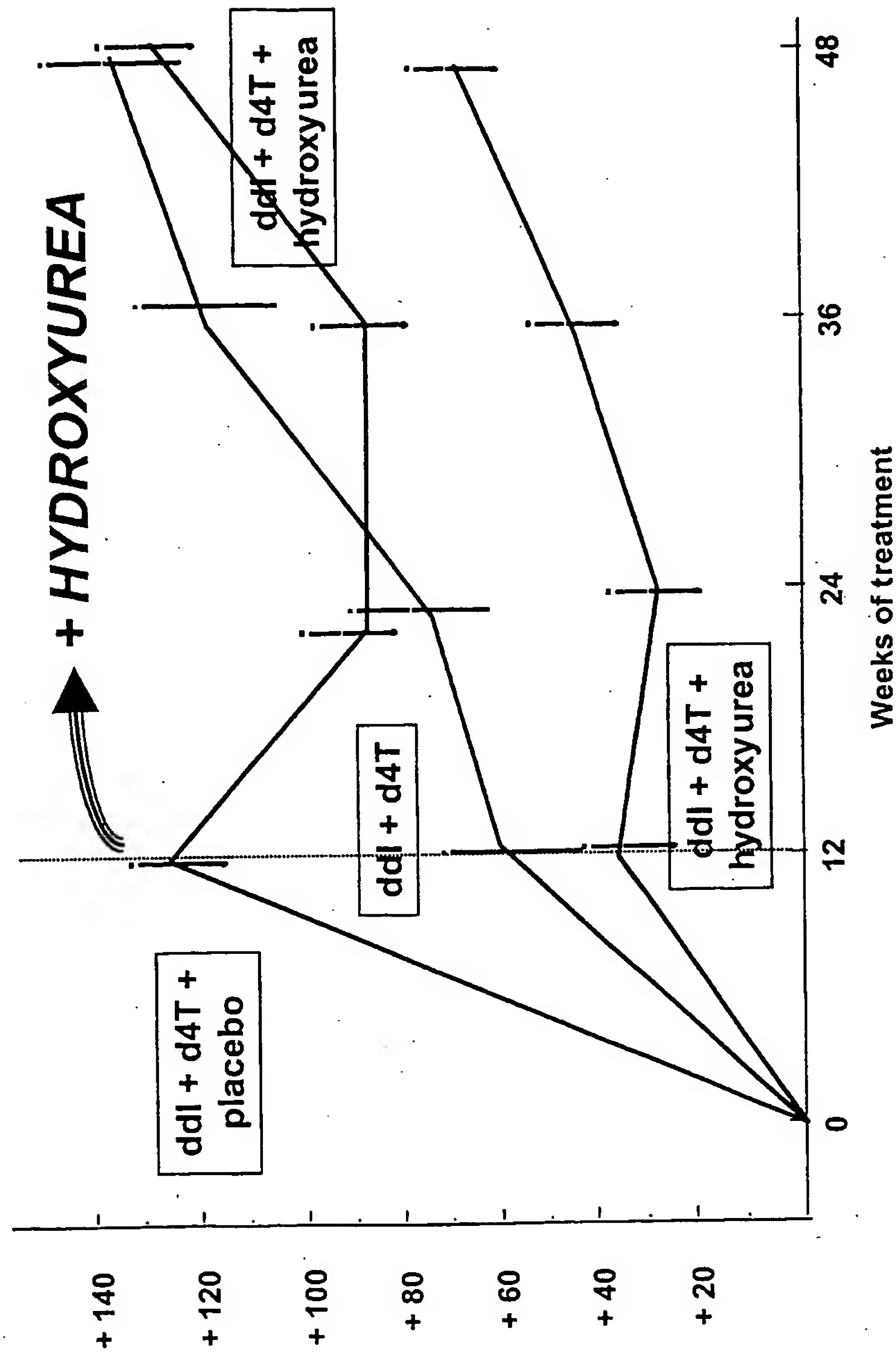
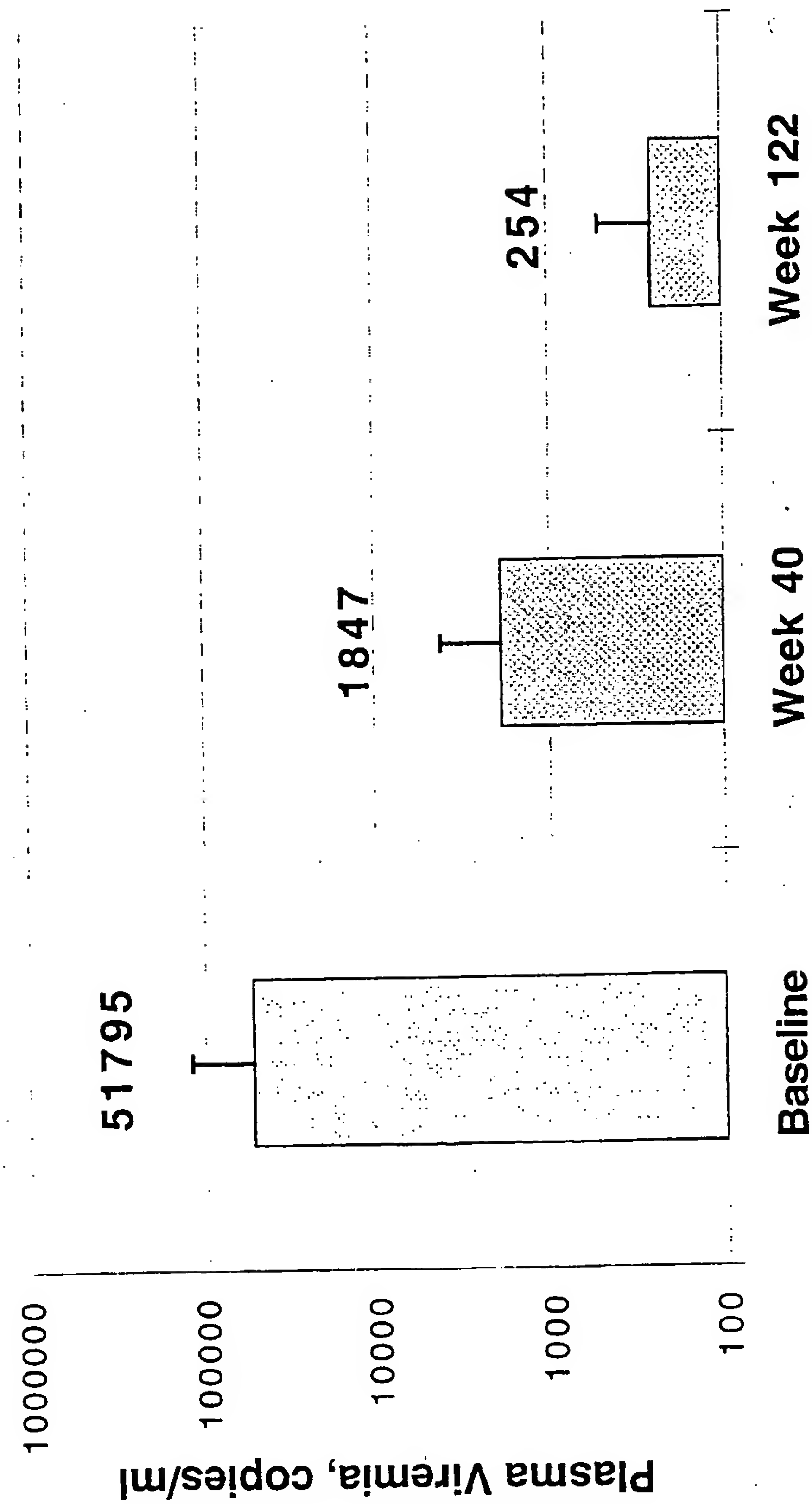
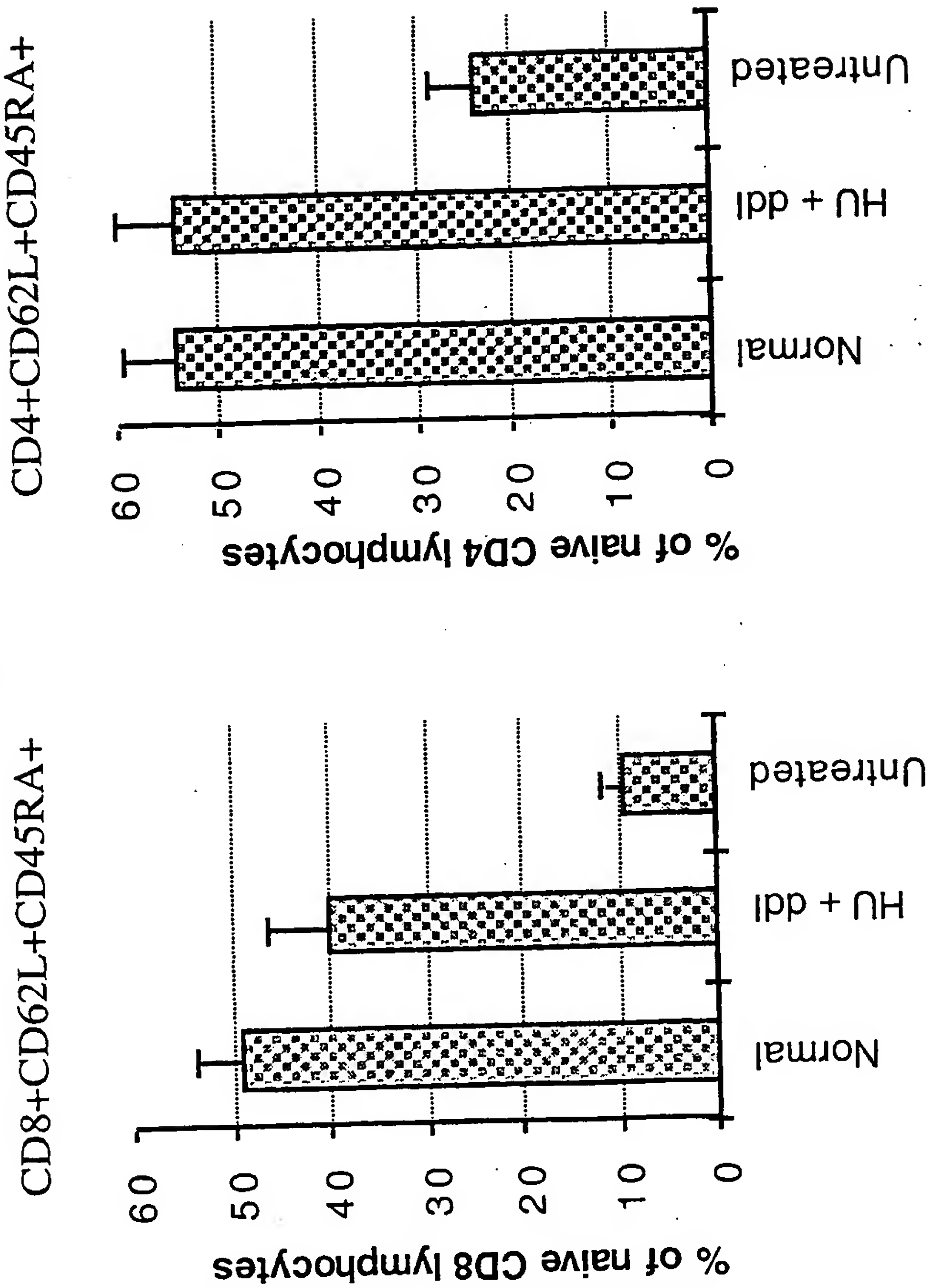


Fig. 11



Plasma Viremia, copies/ml
1000000
100000
10000
1000
100
51795
1847
254
Baseline
Week 40
Week 122

Fig. 12


CD4+CD62L+CD45RA+
% of naive CD4 lymphocytes
60
50
40
30
20
10
0
Normal
HU + ddI
Untreated
CD8+CD62L+CD45RA+
% of naive CD8 lymphocytes
60
50
40
30
20
10
0
Normal
HU + ddI
Untreated

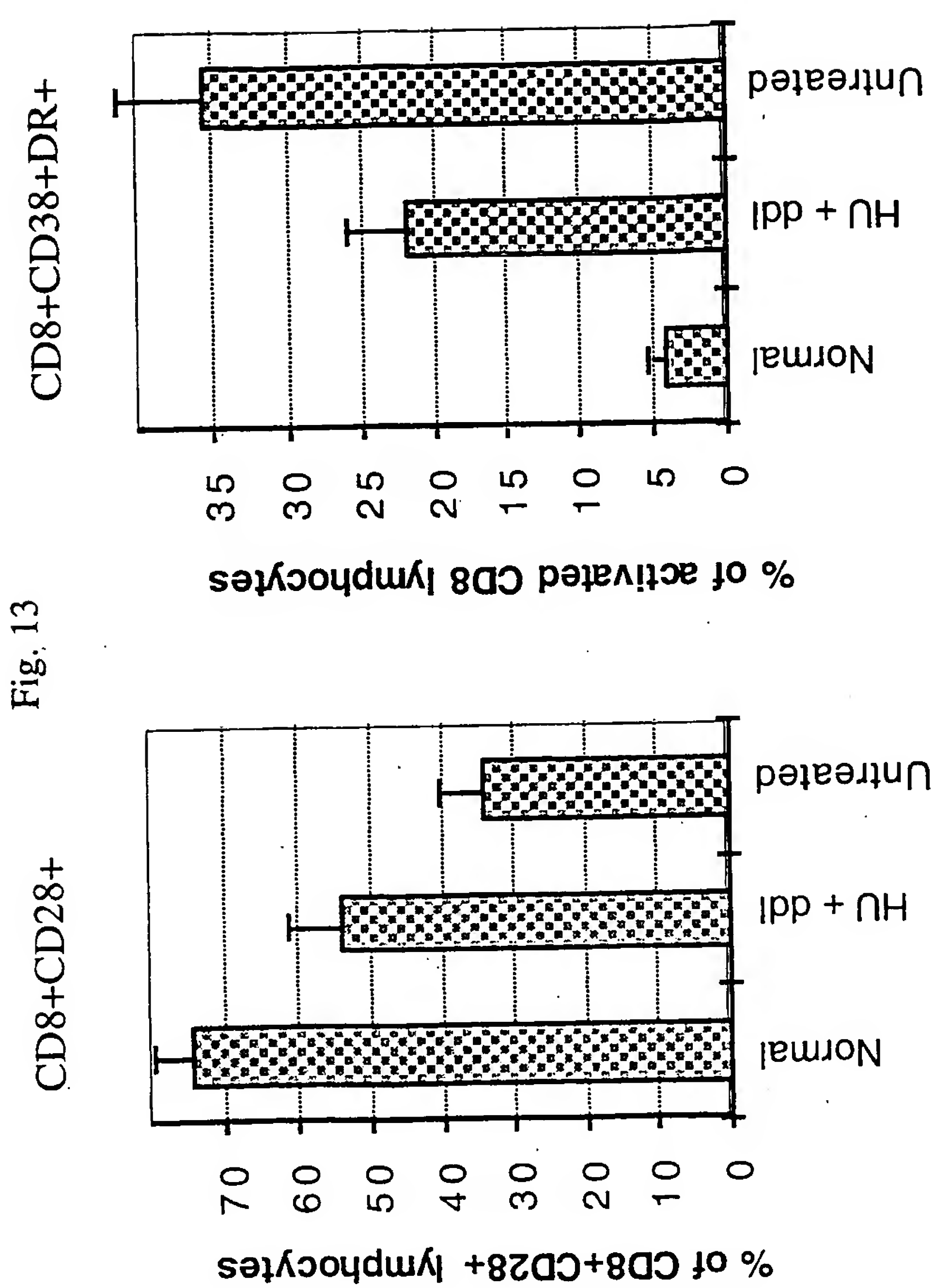

CD8+CD38+DR+
% of activated CD8 lymphocytes
35
30
25
20
15
10
5
0
Untreated
HU + ddI
Normal
CD8+CD28+
% of CD8+CD28+ lymphocytes
70
60
50
40
30
20
10
0
Untreated
HU + ddI
Normal

Fig. 13

Fig. 14

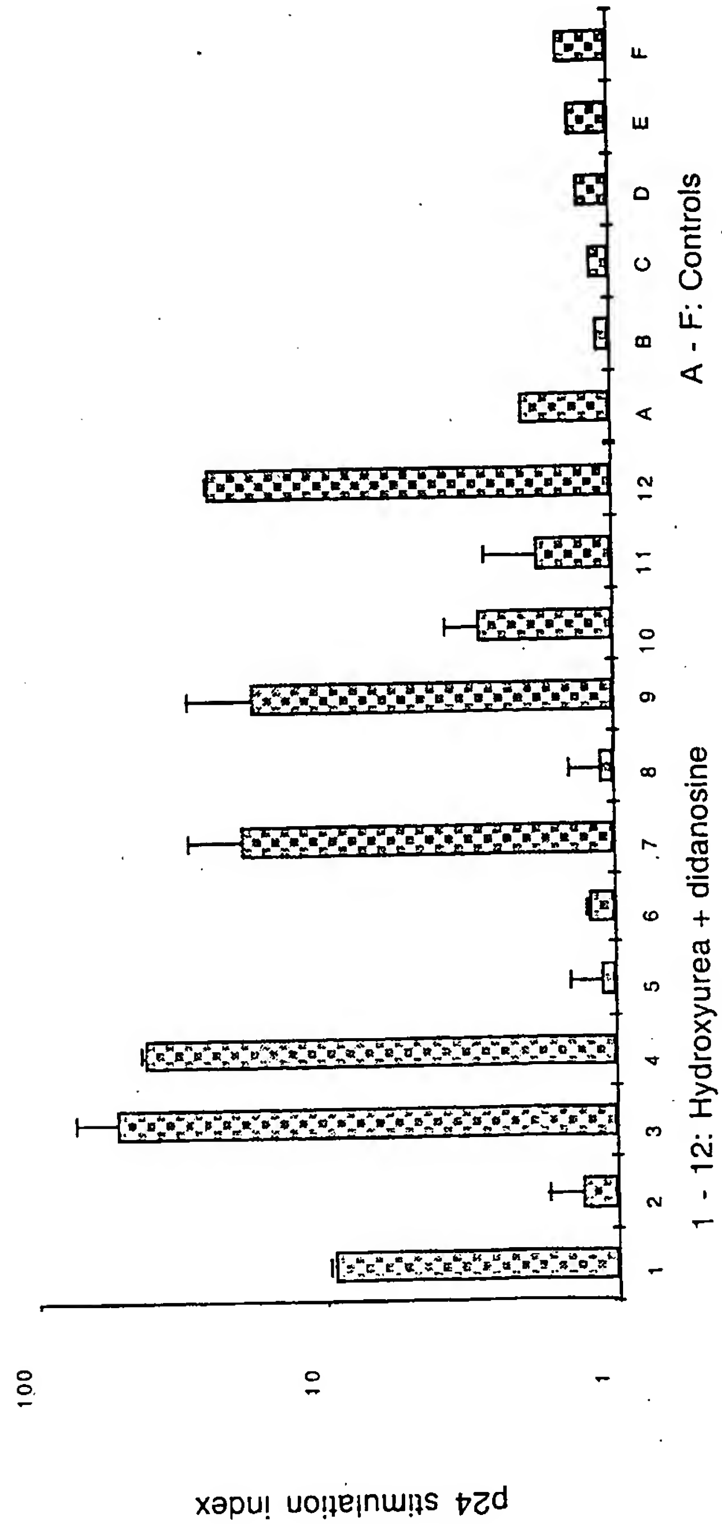

p24 stimulation index
100
10
1
1 2 3 4 5 6 7 8 9 10 11 12 A B C D E F
1 - 12: Hydroxyurea + didanosine
A - F: Controls

Fig. 15

Patient BM
HU + ddI + IDV

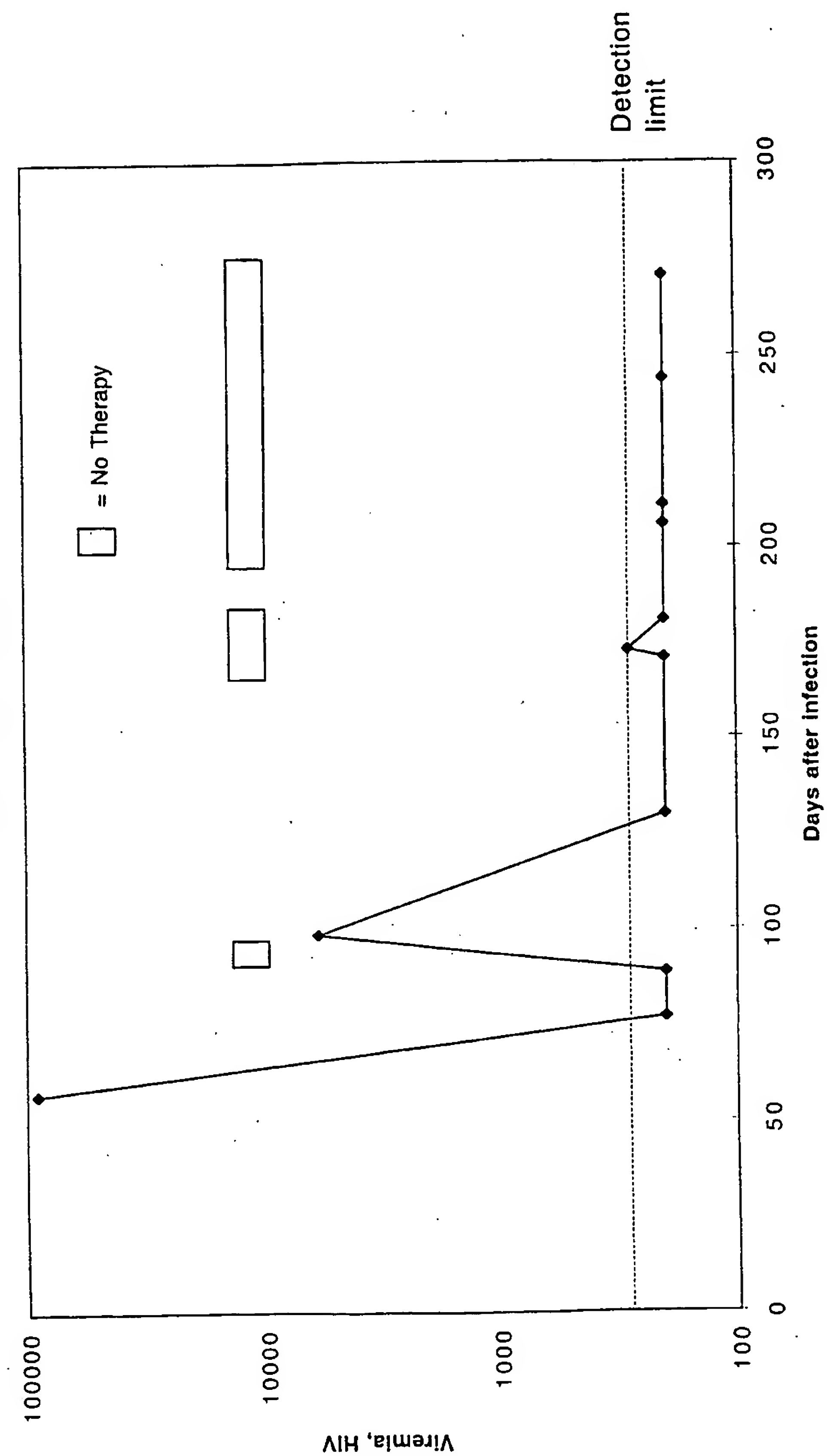


Fig. 16

Animal #1
HU + ddI + PMPA

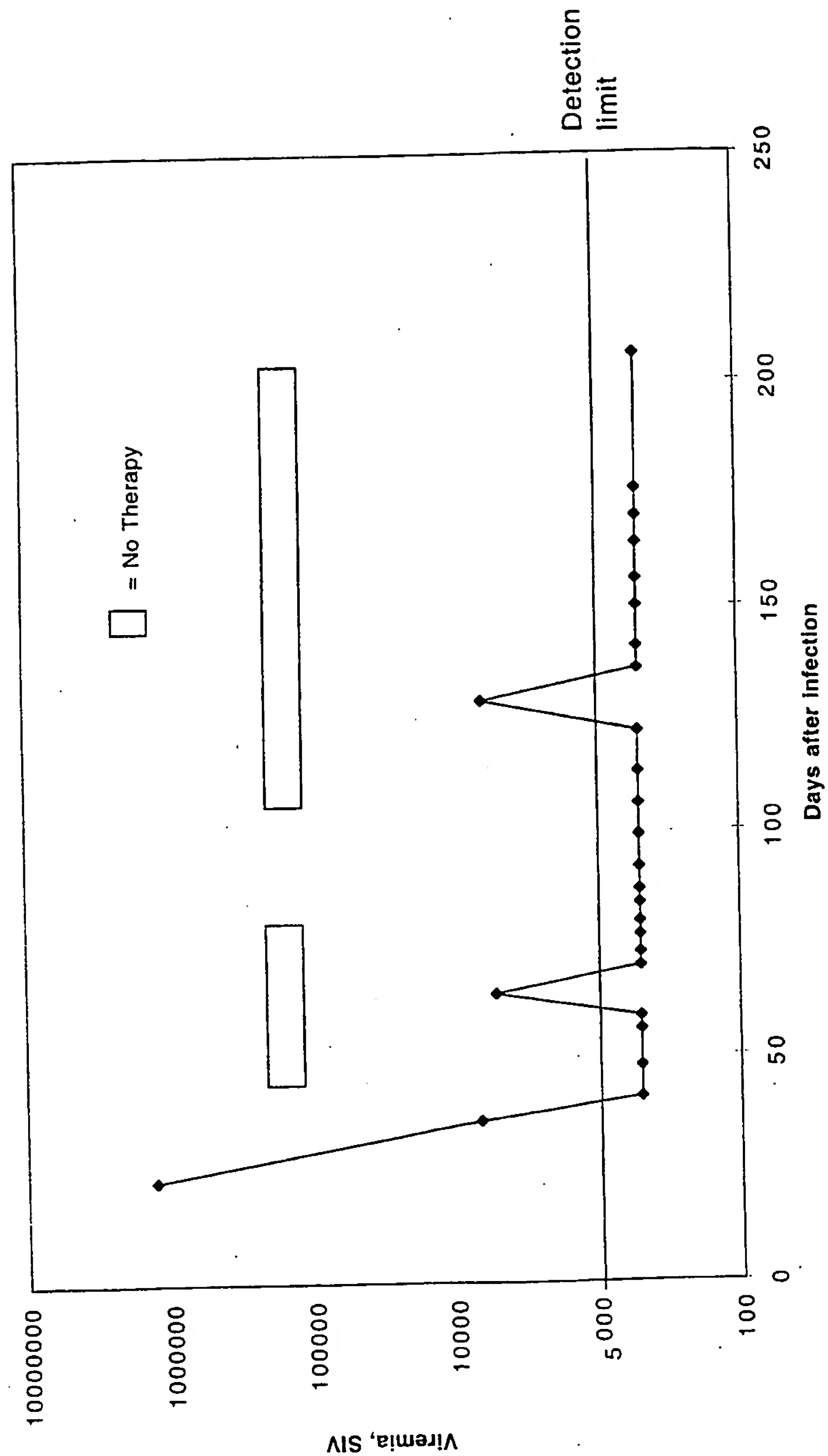


Fig. 17

Animal #2
HU + ddI + PMPA

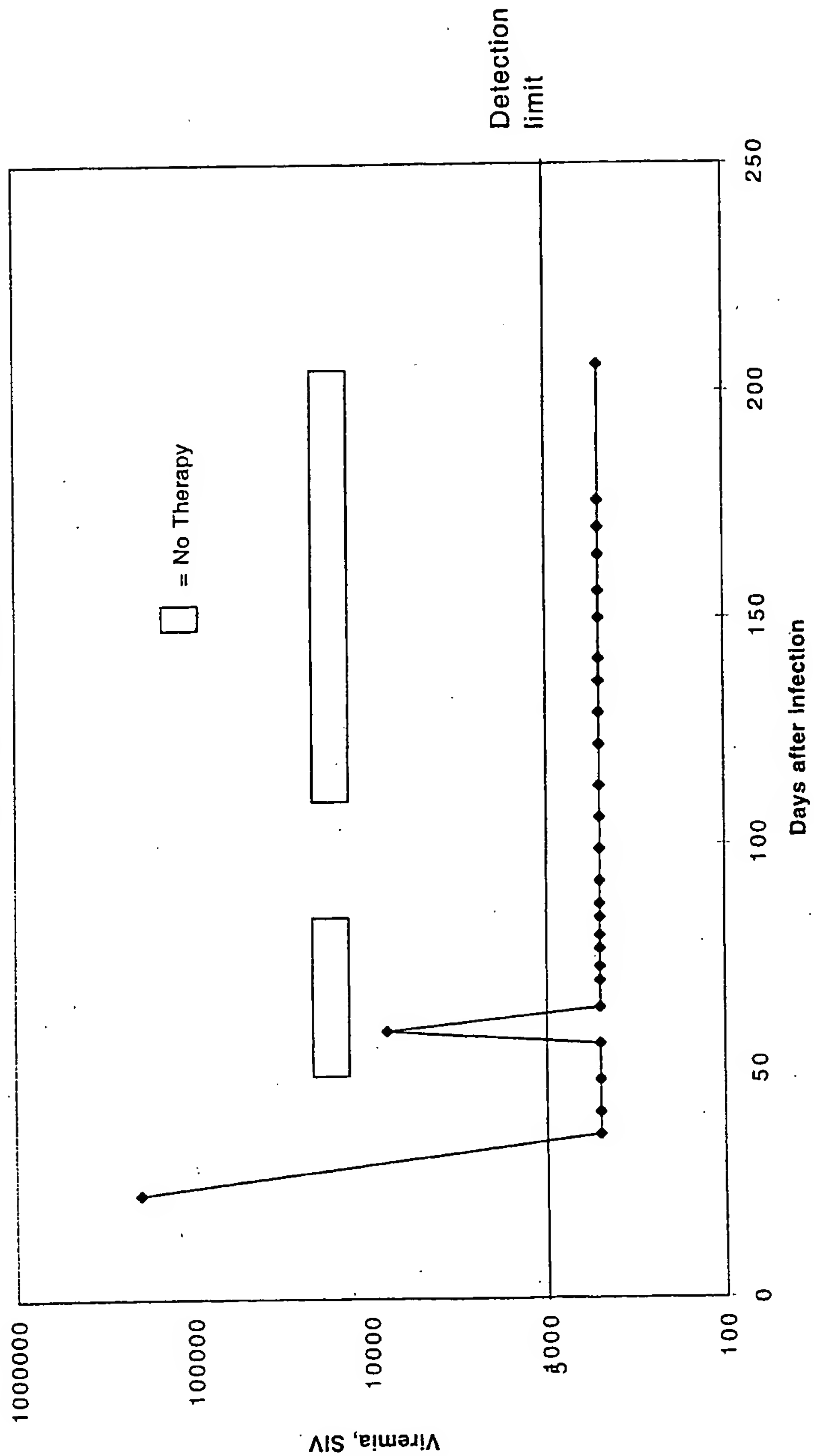


Fig. 18

Animal #3
HU + ddI + PMPA

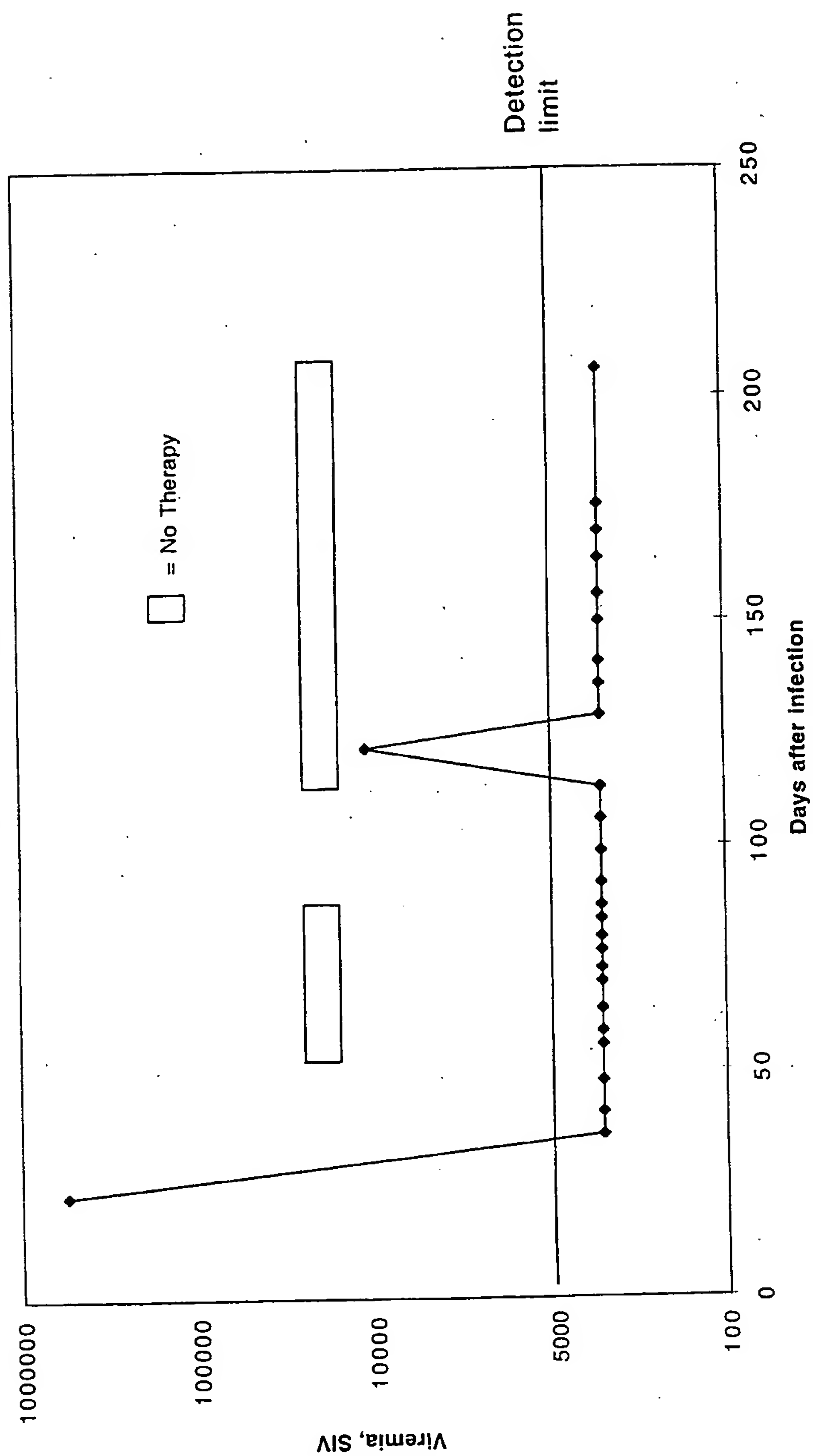


Fig. 19

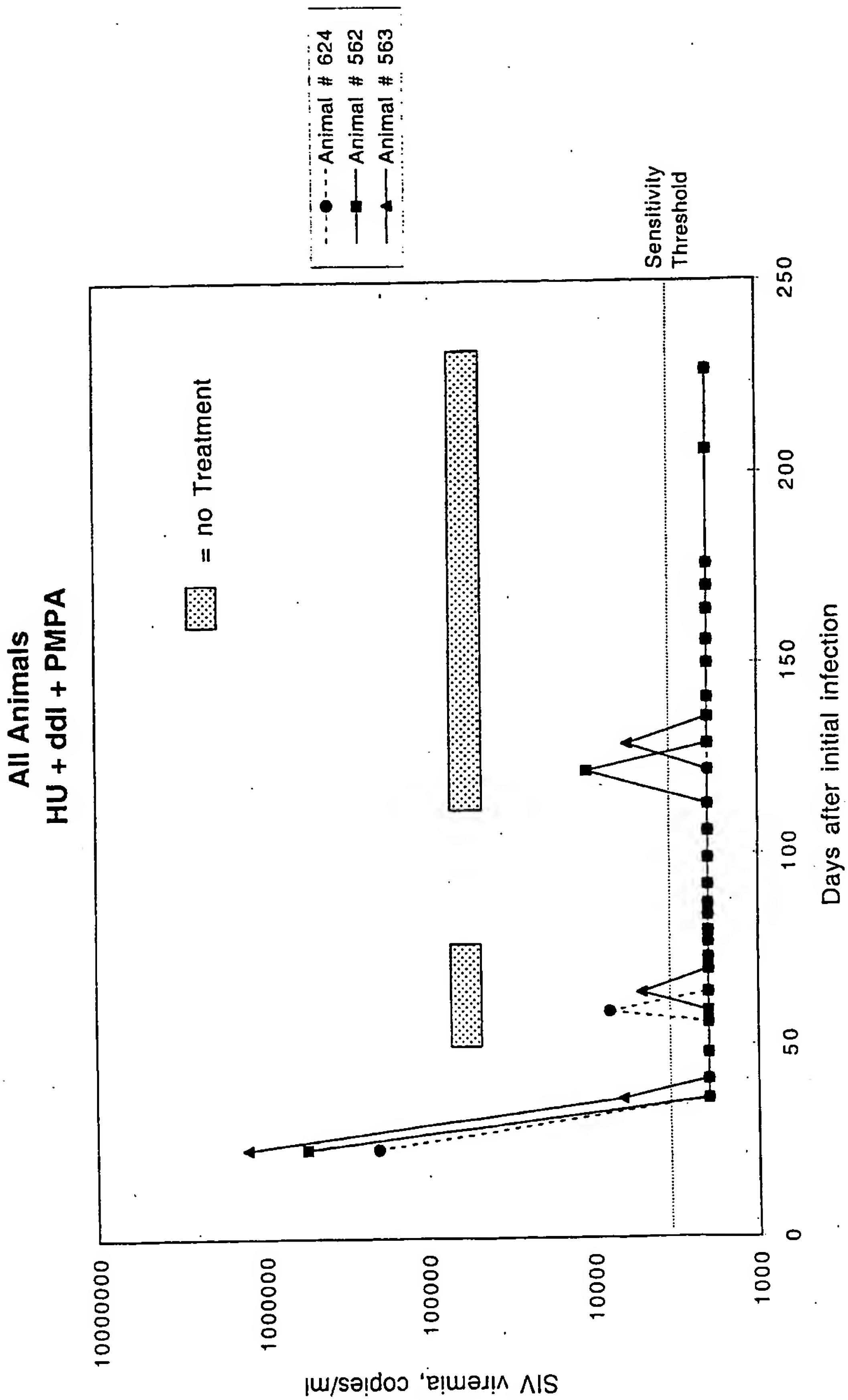

All Animals
HU + ddI + PMPA
= no Treatment
Animal # 624
Animal # 562
Animal # 563
Sensitivity Threshold
SIV viremia, copies/ml
10000000
1000000
100000
10000
1000
0
50
100
150
200
250
Days after initial infection

Fig. 20

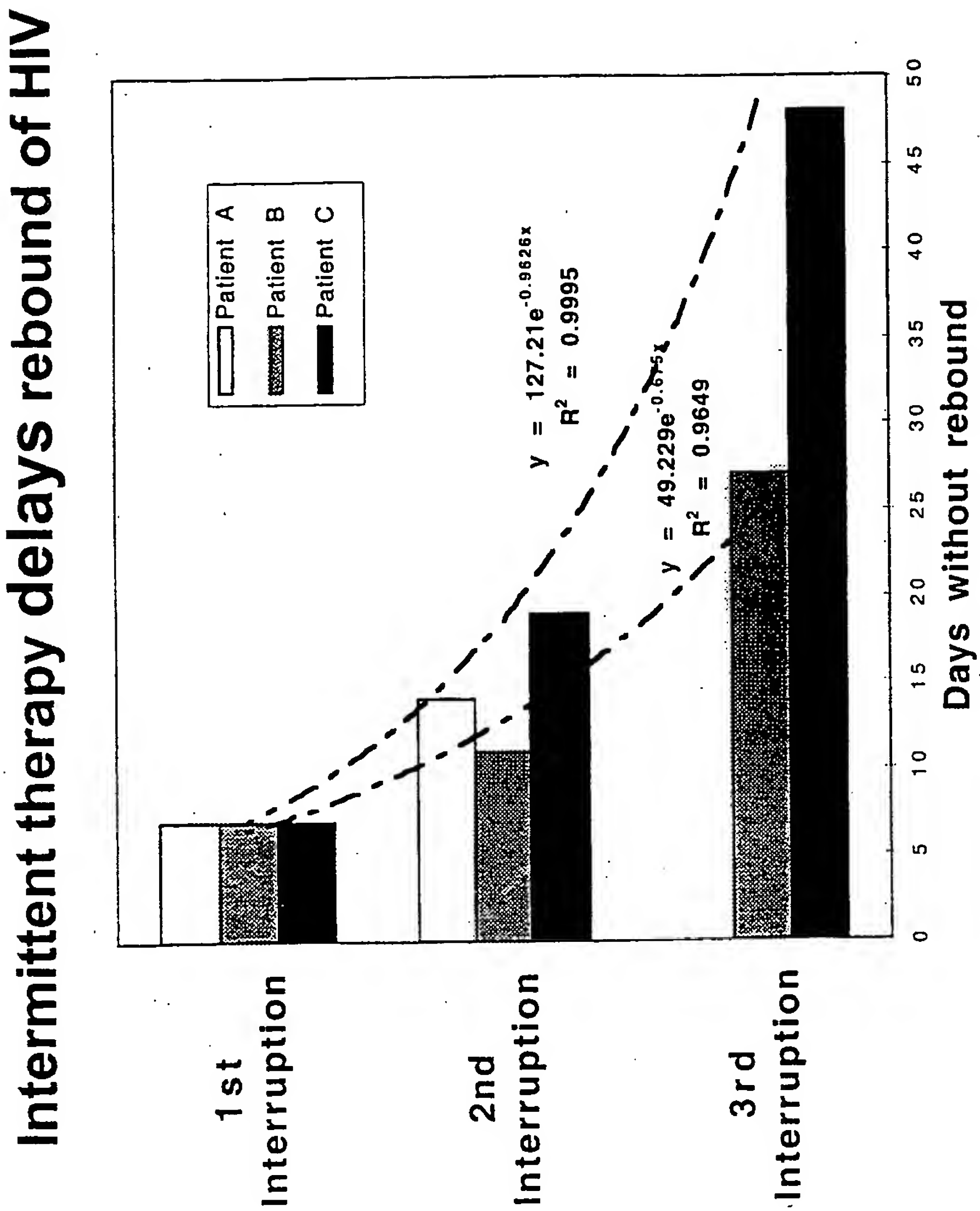

Intermittent therapy delays rebound of HIV
Patient A
Patient B
Patient C
y = 127.21e^-0.9626x
R² = 0.9995
y = 49.229e^0.675x
R² = 0.9649
1 st Interruption
2nd Interruption
3 rd Interruption
0
5
10
15
20
25
30
35
40
45
50
Days without rebound

Fig. 21

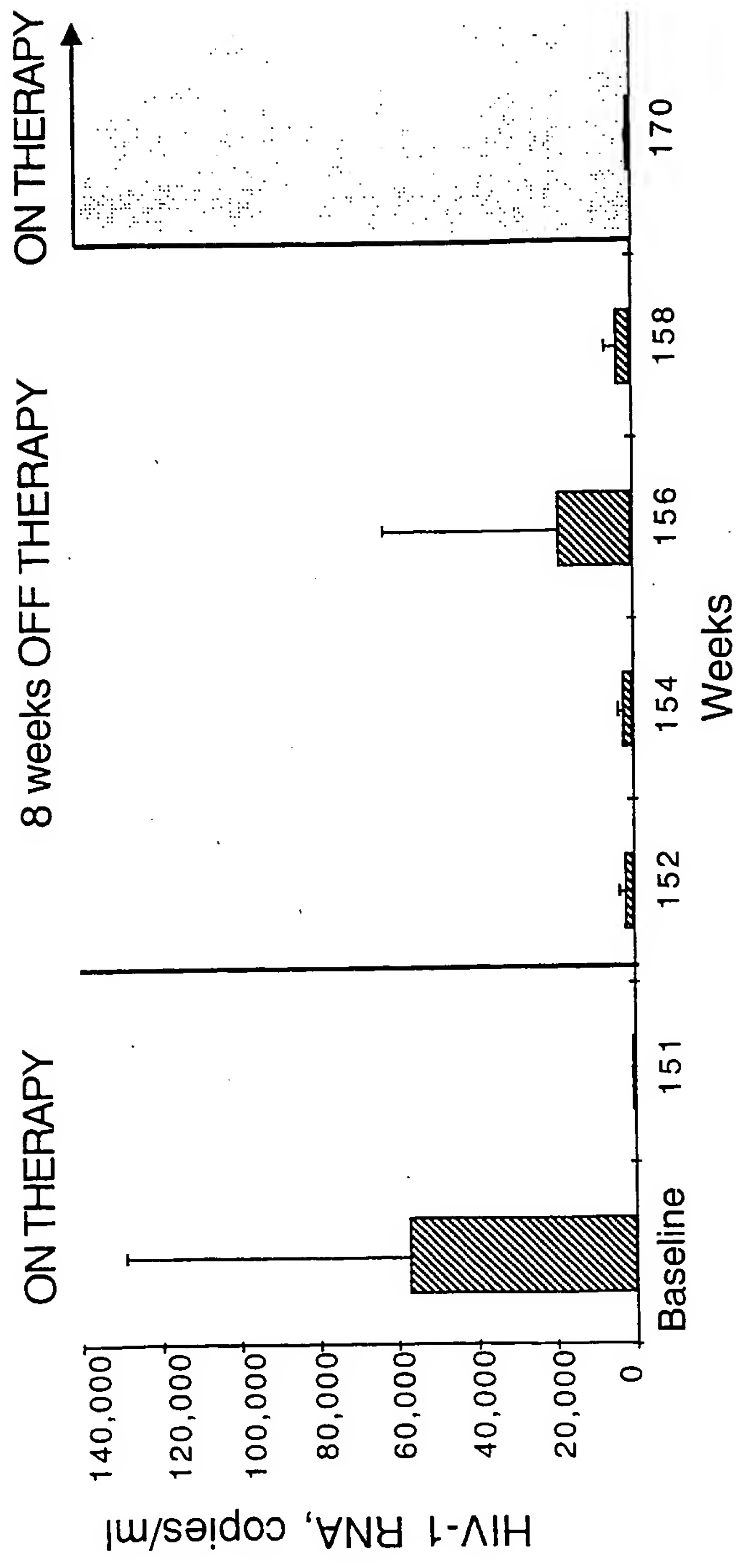

ON THERAPY
8 weeks OFF THERAPY
ON THERAPY
HIV-1 RNA, copies/ml
140,000
120,000
100,000
80,000
60,000
40,000
20,000
0
Baseline
151
152
154
156
158
170
Weeks

Fig. 22

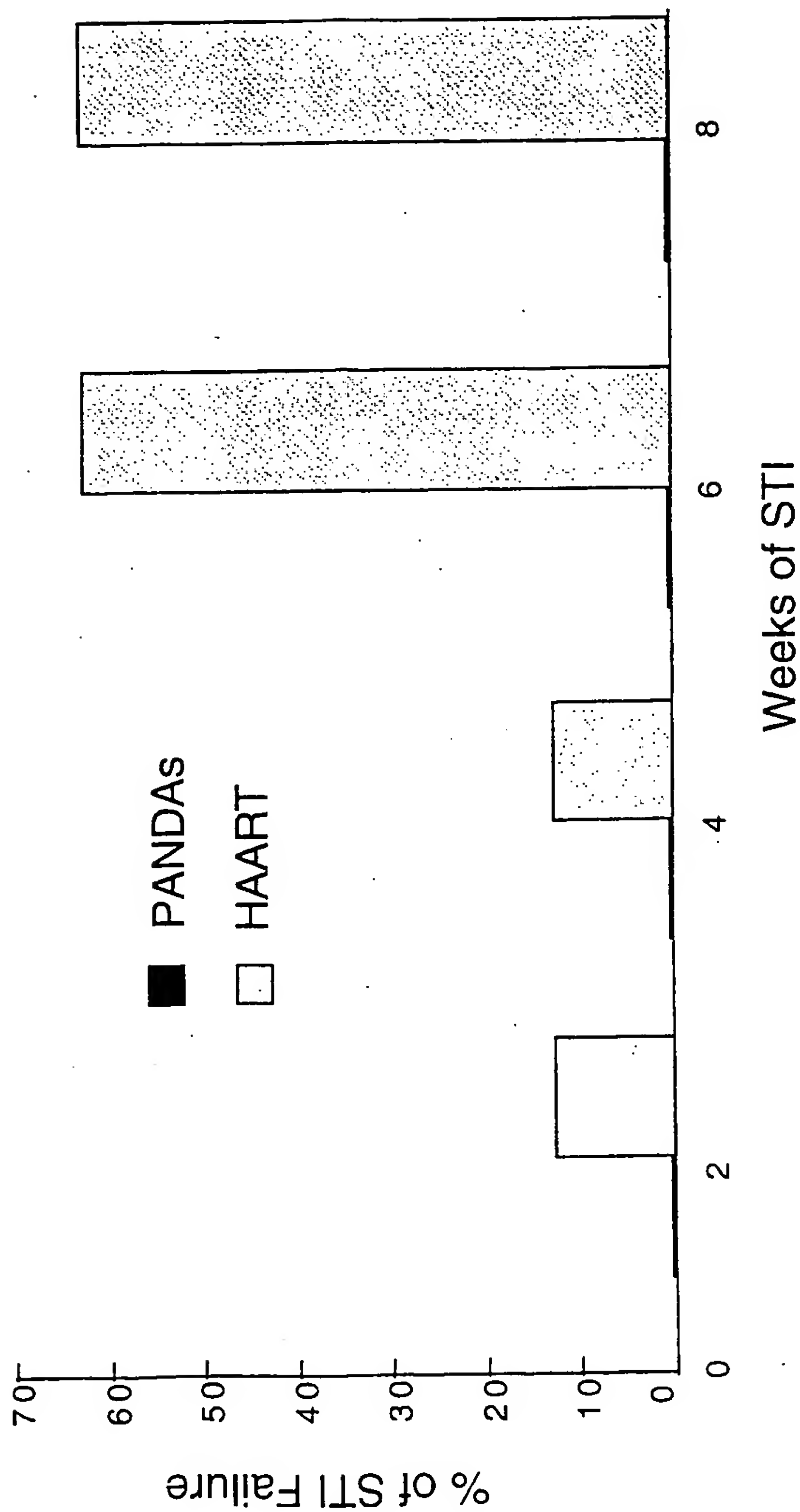

PANDAs
HAART
% of STI Failure
0
10
20
30
40
50
60
70
Weeks of STI
0
2
4
6
8

Fig. 23

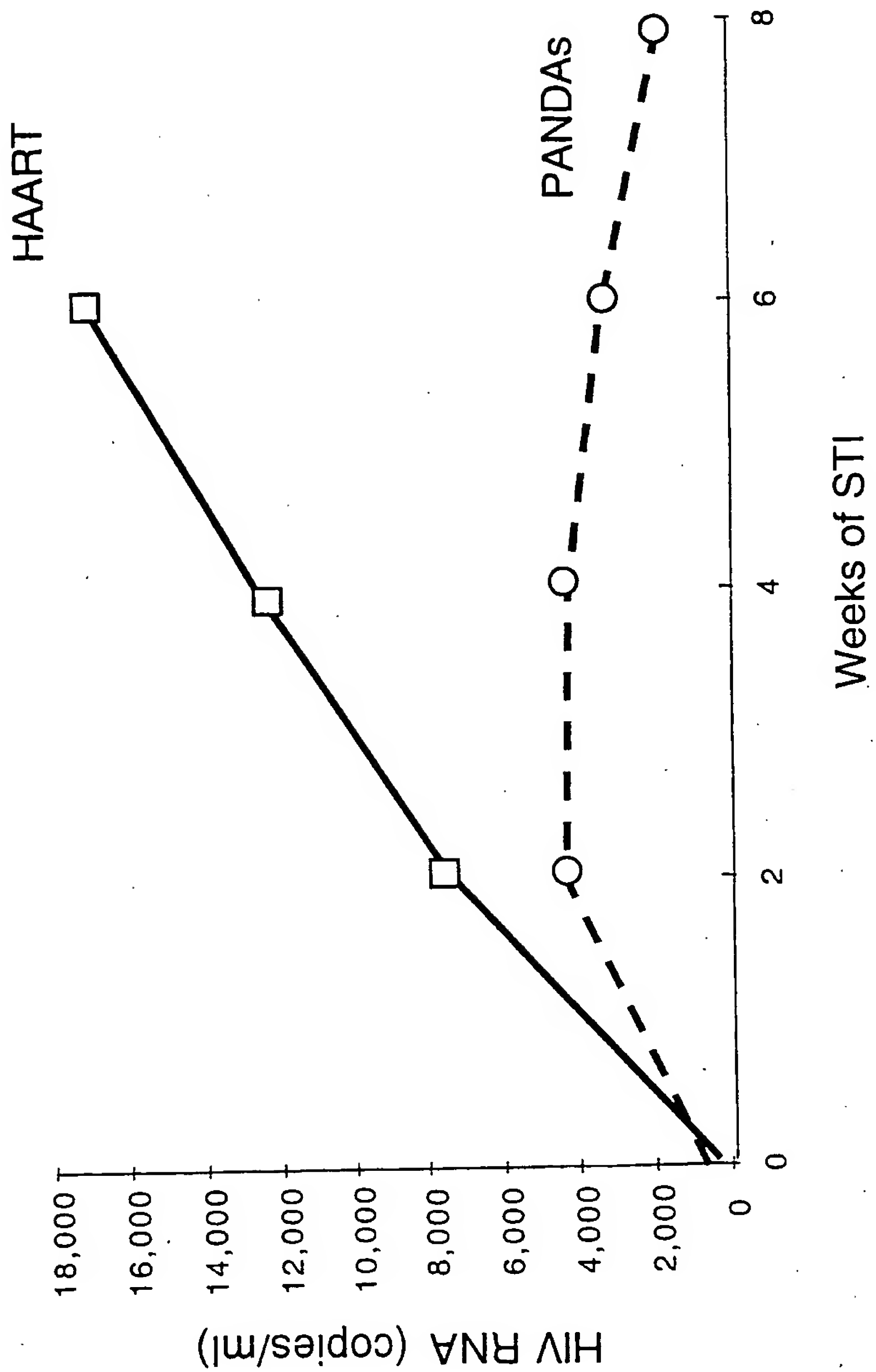
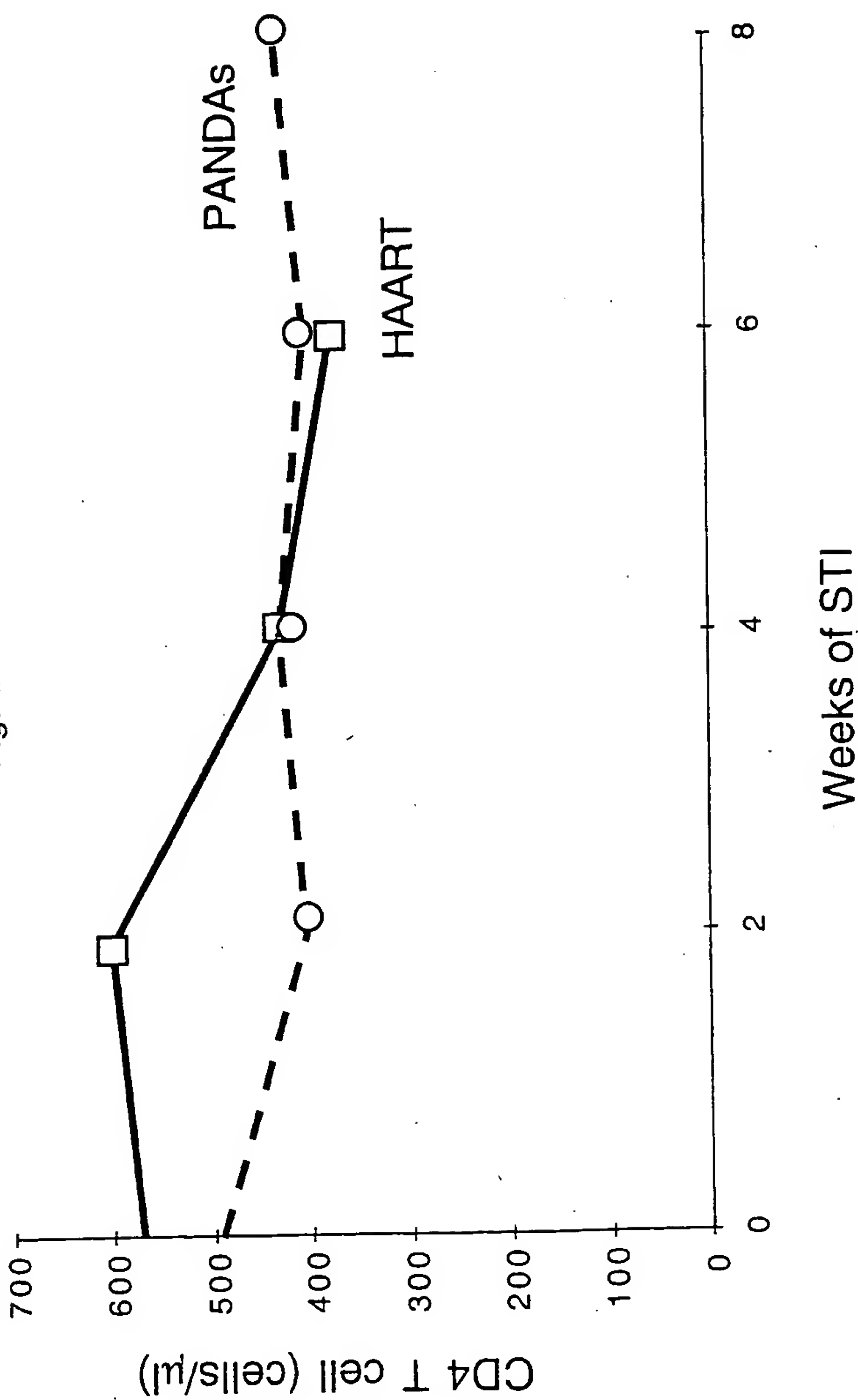

HAART
PANDAs
HIV RNA (copies/ml)
18,000
16,000
14,000
12,000
10,000
8,000
6,000
4,000
2,000
0
0
2
4
6
8
Weeks of STI

Fig. 24


PANDAs
HAART
CD4 T cell (cells/µl)
700
600
500
400
300
200
100
0
0
2
4
6
8
Weeks of STI

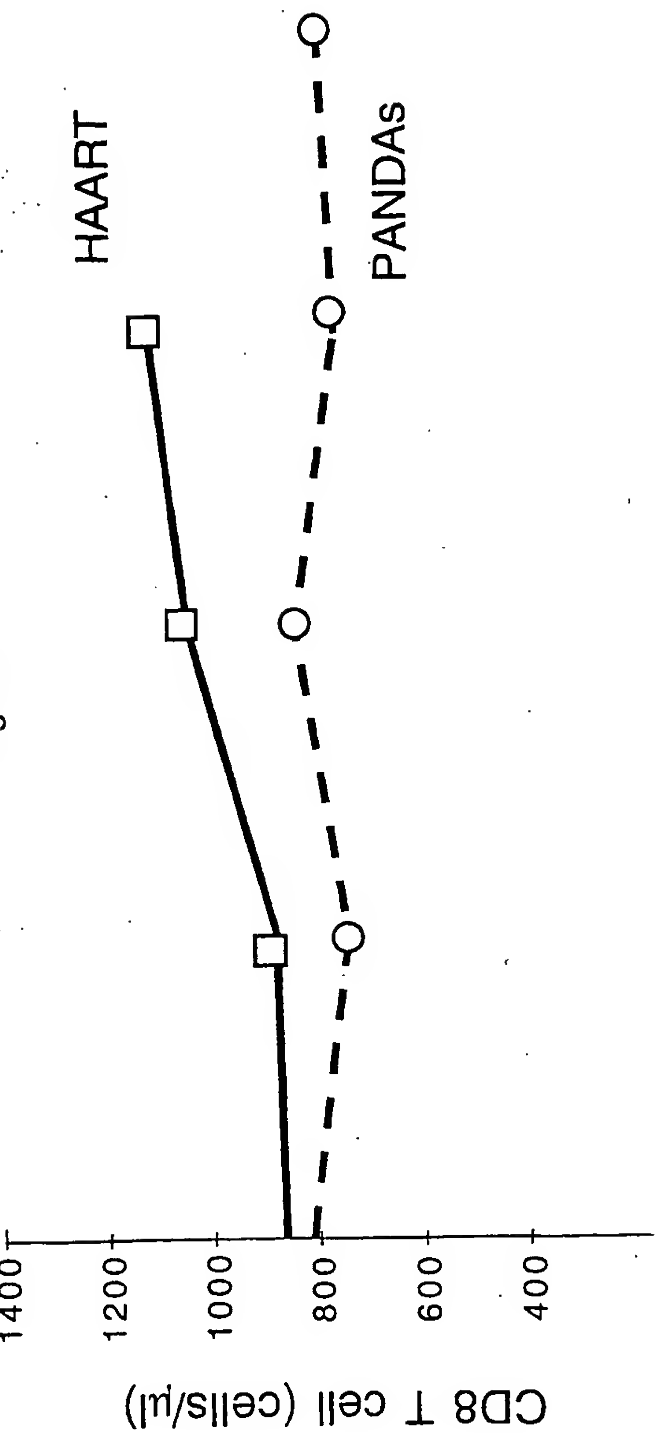


Fig. 25

Fig. 26

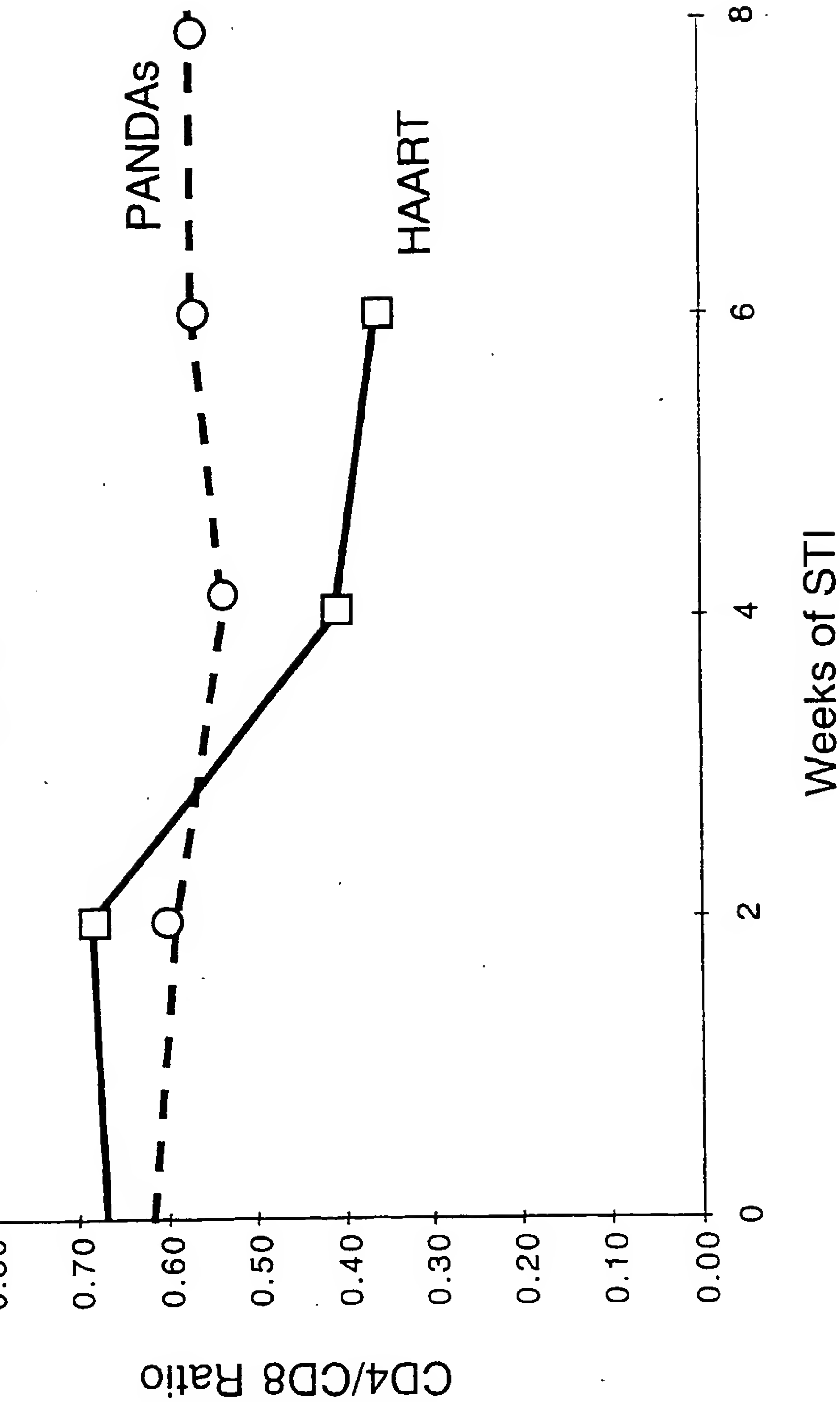

PANDAs
HAART
CD4/CD8 Ratio
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
2
4
6
8
Weeks of STI

Fig. 27

STI in an SIV-infected monkey model

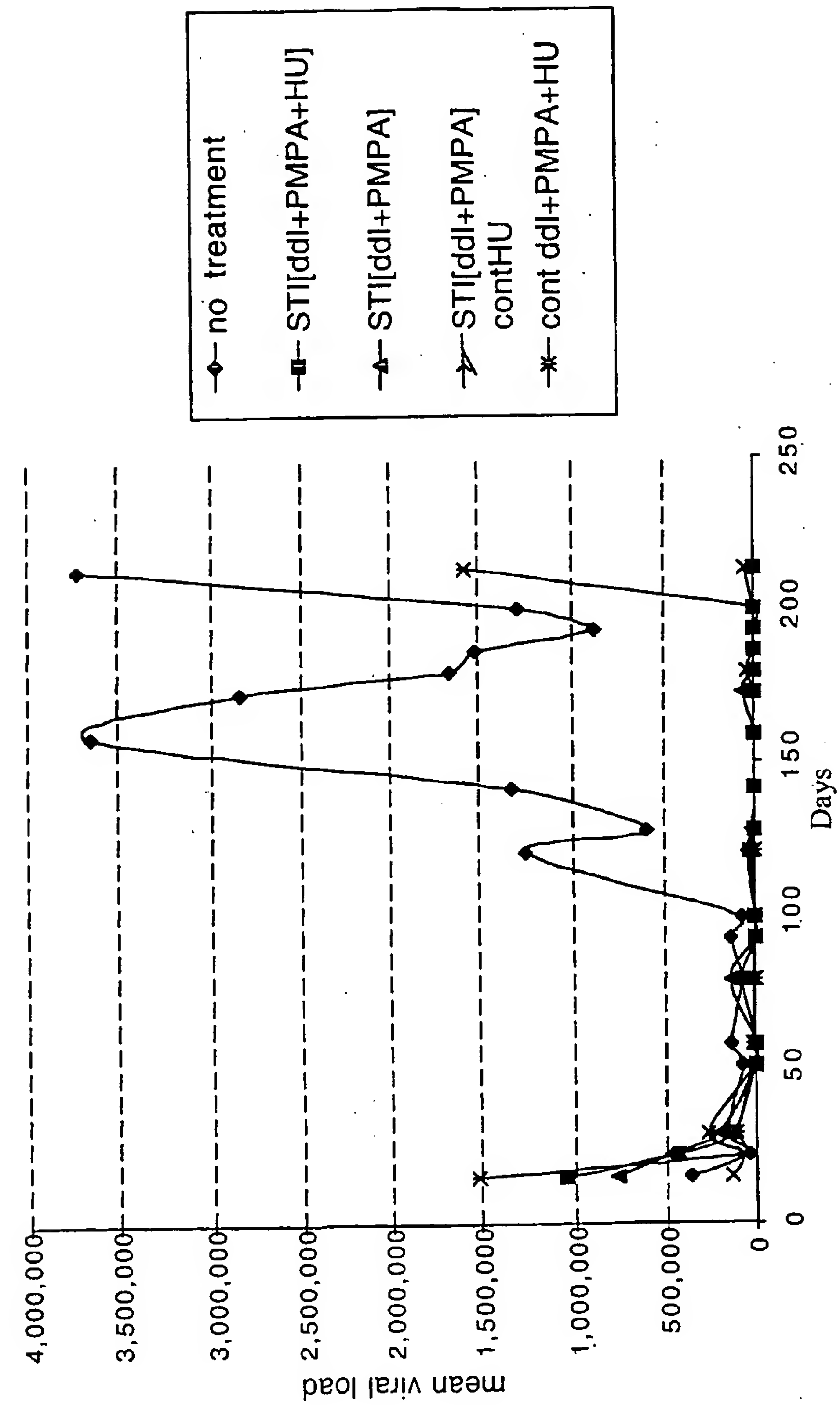


Fig. 28

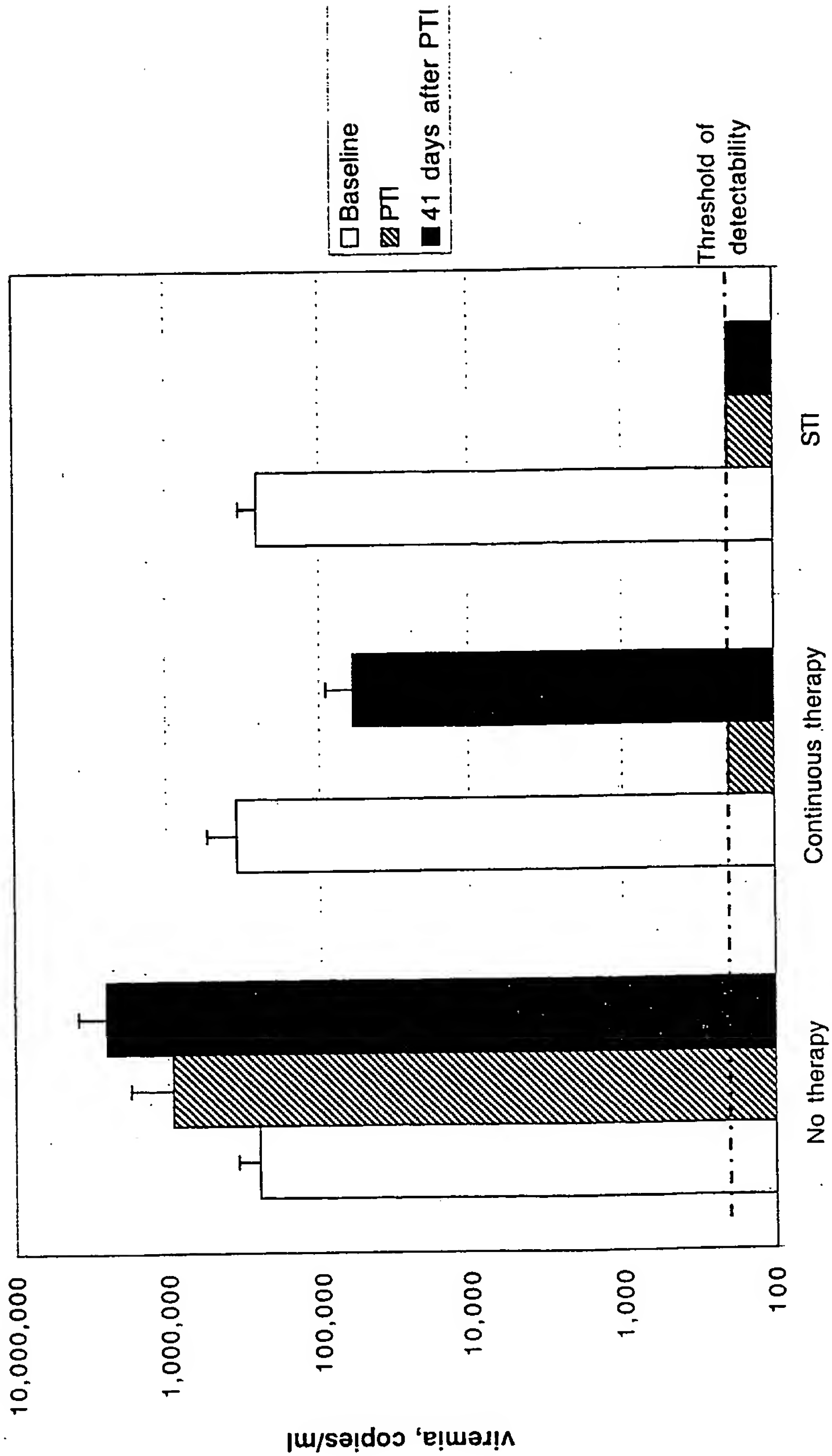
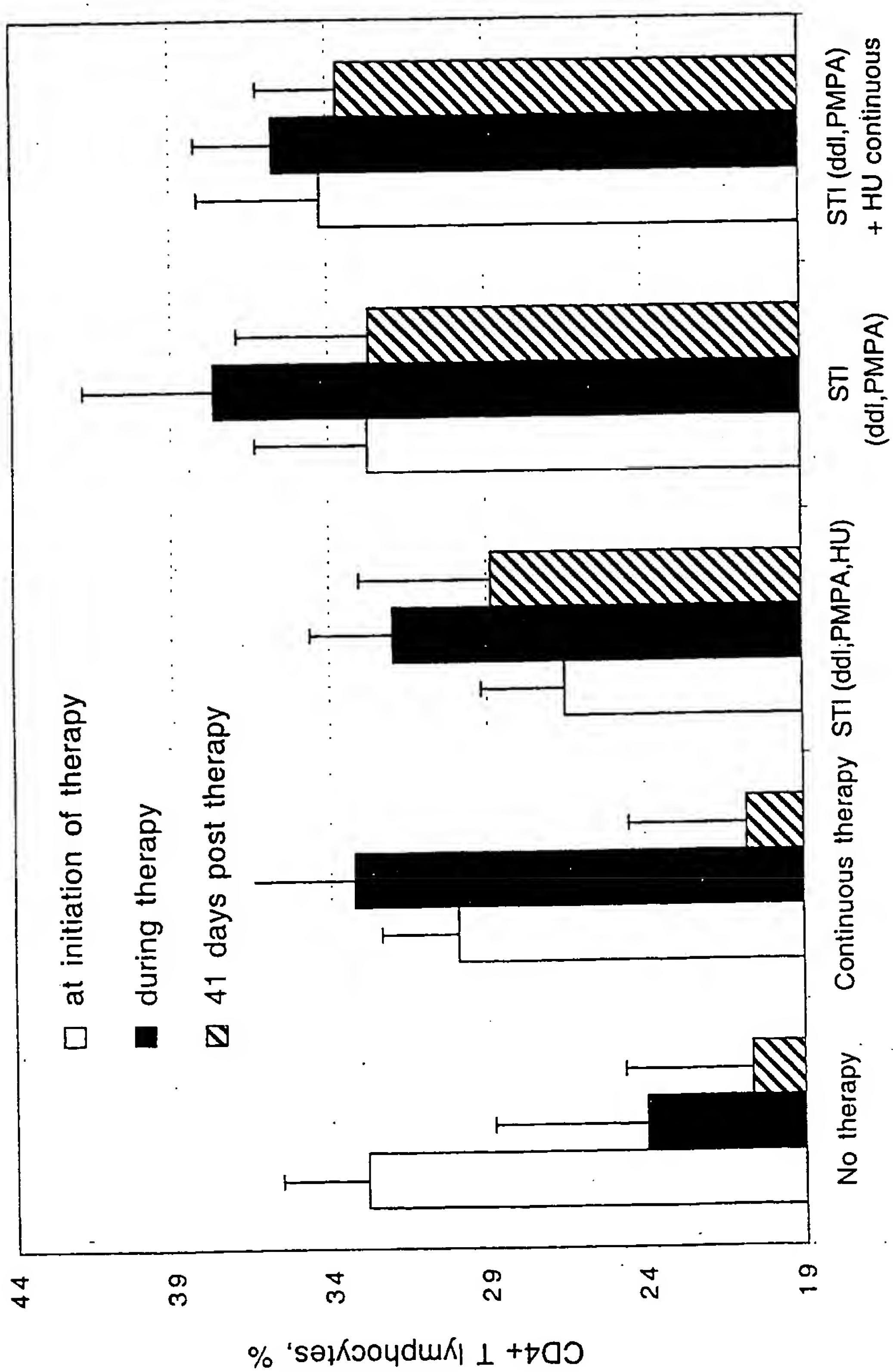

Baseline
PTI
41 days after PTI
Threshold of detectability
No therapy
Continuous therapy
STI
10,000,000
1,000,000
100,000
10,000
1,000
100
viremia, copies/ml

Fig. 29


at initiation of therapy
during therapy
41 days post therapy
CD4+ T lymphocytes, %
44
39
34
29
24
19
No therapy
Continuous therapy
STI (ddI,PMPA,HU)
STI (ddI,PMPA)
STI (ddI,PMPA) + HU continuous

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Lisziewicz, Julianna
(B) STREET: 4400 East-West Highway Apt. 1126
(C) CITY: Bethesda
(D) STATE: Maryland
(E) COUNTRY: US
(F) POSTAL CODE (ZIP): 20814
(G) TELEPHONE: 202 687 2833
(H) TELEFAX: 202 687 2907
(I) TELEX: n/a

(A) NAME: Lori, Franco
(B) STREET: 44 East-West Highway Apt. 1126
(C) CITY: Bethesda
(D) STATE: Maryland
(E) COUNTRY: US
(F) POSTAL CODE (ZIP): 20814
(G) TELEPHONE: 202 687 2833
(H) TELEFAX: 202 687 2907
(I) TELEX: n/a

(ii) TITLE OF INVENTION: Method of Inhibiting Human Immunodeficiency Virus by combined use of Hydroxyurea, a nucleoside analog, and a protease inhibitor

(iii) NUMBER OF SEQUENCES: 6

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(v) CURRENT APPLICATION DATA:
APPLICATION NUMBER: US 08/812515

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Human immunodeficiency virus type 1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GGACCTACAC CTGTCAACAT 20

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Human immunodeficiency virus type 1

(viii) POSITION IN GENOME:
(B) MAP POSITION: 886 to 908 of HXB2 pol gene
(C) UNITS: bp

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

CATTTATCAG GATGGAGTTC ATA 23

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GGATGGAAAG GATCACCAGC 20

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Human immunodeficiency virus type 1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

TACTAGGTAT GGTAAATGCA GT 22

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Human immunodeficiency virus type 1

(viii) POSITION IN GENOME:
(B) MAP POSITION: 233 to 254 of HXB2 pol gene
(C) UNITS: bp

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

CAGGAATGGA TGGCCCAAAA GT 22

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Human immunodeficiency virus type 1

(viii) POSITION IN GENOME:
(B) MAP POSITION: 874 to 891 of HXB2 pol gene
(C) UNITS: bp

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

TTCATAACCC ATCCAAAG 18

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/02754

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :A61K 39/42, 39/00, 39/21, 39/29, 39/12, 39/13, 31/70
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/160.1, 161.1, 185.1, 188.1, 189.1, 216.1, 217.1; 514/45, 46, 47, 48, 49, 50, 51.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

File CA structure search in parent case, US Appl. SN 09/048,576.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,977,086 A (LISZIEWICZ et al.) 02 November 1999, see claims 1-7 in particular.	1-3 and 5-7
Y	US 5,521,161 A (MALLEY et al.) 28 May 1996, see Figures 1 and 3 and claims 1-3 in particular.	1-7
Y	VILA et al. Absence of Viral Rebound After Treatment of HIV-Infected Patients with Didanosine and Hydroxycarbamide. Lancet. 30 August 1997, Vol. 350, pages 635-636, see entire document.	1-7
Y	US 5,413,999 A (VACCA et al.) 09 May 1995, see abstract and end of column 57 in particular.	8-11

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 20 MARCH 2000	Date of mailing of the international search report 06 JUN 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer LAWRENCE ERIC CRANE Telephone No. (703) 308-1235

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/160.1, 161.1, 185.1, 188.1, 189.1, 216.1, 217.1; 514/45, 46, 47, 48, 49, 50, 51.